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Low temperature and leaf growth in grasses

By

Aidan D Farrell

Thesis submitted for degree of Ph.D.

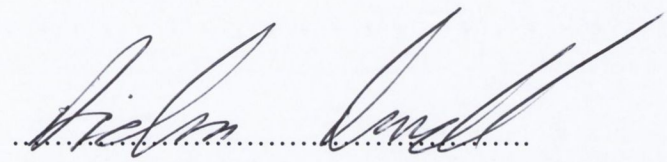
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Summary

The central role of temperature in regulating plant growth was investigated by analysing the temperature sensitivity of various aspects of growth in grass leaves.

A study of thermal responses in the C_4 grass *Miscanthus* found significant genotypic variation in the effect of temperature on shoot emergence, leaf extension and seedling survival. One genotype, Sin-H9, exhibited low temperature sensitivity in leaf extension rate and shoot emergence rate. Shoots of Sin-H9 also had the highest frost tolerance, which was associated with significantly lower shoot moisture content in this genotype. The reduced time to emergence and increased frost tolerance seen in Sin-H9 was incorporated into a model of *Miscanthus* productivity using climate data from northern Europe. The longer growing season was predicted to increase yields by up to 26 %.

The productivity model also highlighted the importance of the short-term thermal response of leaf extension rate in determining yield over the whole season. In subsequent studies the mechanism through which temperature effects leaf extension rate was examined by analysing the effect of cooling on cell turgor pressure and cell wall properties.

Cooling of the leaf extension zone was shown to cause an immediate reduction in the leaf extension rate of maize and barley seedlings. This inhibition was not associated with a drop in turgor pressure in the expanding cells. When growing leaves were artificially provided with extra force in the direction of growth leaf extension was increased but the inhibitory effects of cooling remained, indicating that reduced turgor pressure was not the point of limitation.

In vivo measurements showed that cooling caused a marked drop in the extensibility of the growing cell walls. The reduced extensibility was not associated with a change in the mechanical properties of the walls. *In vitro* measurements were also carried out using freeze/thawed tissue from the maize leaf extension zone held under constant force. The extension of such tissue was found to respond immediately to cooling, with a thermal response similar to that seen *in vivo*. The response to temperature was lost in tissue that was heat-inactivated prior to testing.

Taken together the results support the hypothesis that short-term effects of temperature on leaf extension are mediated through reduced activity of wall loosening proteins in the expanding cells.

Although short-term cooling did not affect the mechanical properties of wall tissue, differences between genotypes in the thermal response of leaf extension were associated with differences in mechanical wall properties. This illustrates that the leaf extension rate at any given temperature is a net result of the two factors and suggests that differences in wall structure can modulate the extent to which loosening is reduced during cooling.

Table of content

Acknowledgements i

Summary ii

Table of content iii

List of abbreviations and terminology vi

Chapter 1: An Introduction to temperature and plant growth

1.1 Temperature and plant growth 1
- 1.1.1 Temperature and growth limiting processes
  - Box 1.1 Chilling and freezing damage in plants
- 1.1.2 Carbohydrate supply at suboptimal temperatures
- 1.1.3 Translocation of carbon at suboptimal temperatures

1.2 Temperature and leaf extension 10
- 1.2.1 The process of cell expansion
- 1.2.2 The Lockhart equation
- 1.2.3 Cell expansion and turgor pressure
- 1.2.4 Cell expansion and cell wall properties
  - Box 1.2 The growing cell wall
  - Box 1.3 Measuring cell wall rheology
- 1.2.5 Wall loosening factors

1.3 Conclusion 22

Chapter 2: Frost tolerance and thermal response of leaf growth in four *Miscanthus* genotypes. The impact of genotypic variation on potential yield.

2.1 Introduction 27

2.2 Materials and methods 28
- 2.2.1 Plant material
- 2.2.2 Field site
- 2.2.3 Shoot emergence
- 2.3.4 Frost tolerance
- 2.3.5 Moisture content and osmotic potential
- 2.3.6 Modelling potential yield

2.3 Results 32
- 2.3.1 Shoot emergence
- 2.3.2 Frost tolerance
- 2.3.3 Modelling potential yield

2.4 Discussion 34

Chapter 3: Biophysical control of leaf extension at low temperatures

3.1 Introduction 52

3.2 Materials and methods 55
3.2.1 Plant material
3.2.2 Determining the position of the LEZ
3.2.3 Leaf extension measurements
3.2.4 Pressure Probe Technique
3.2.5 Creep measurements

3.3 Results 59
3.3.1 Response to cooling
3.3.2 Turgor pressure
3.3.3 Creep analysis

3.4 Discussion 62
3.4.1 Water entry, turgor pressure and cell expansion
3.4.2 Creep analysis

Chapter 4. *In vivo* measurement of cell wall properties, in grasses with contrasting thermal responses in leaf extension rate

4.1 Introduction 76

4.2 Materials and methods 78
4.2.1 Maize
4.2.2 Barley
4.2.3 Leaf extension zone cooling
4.2.4 Leaf extension measurements
4.2.5 Applied force

4.3 Results 81
4.3.1 Maize
4.3.2 Barley

4.4 Discussion 82

Chapter 5. Final Discussion

5.1 Introduction 98
5.1.1 Main findings of thesis
5.1.2 Aims of discussion

5.2 Temperature, leaf area and yield 100
5.2.1 Temperature and season length: the role of breeding
5.2.2 Selecting for early emergence

5.3 Temperature and leaf extension 103

5.3.1 The thermal response
5.3.2 Adaptation
5.3.4 Acclimation

5.4 Temperature and turgor pressure **112**
5.4.1 Leaf extension and root cooling
5.4.2 Root cooling and evaporative demand

5.5 Temperature and the cell wall **115**
5.5.1 Cell wall extensibility measurement and meaning
5.5.2 Inhibition of wall expansion during short-term cooling
5.5.3 Inhibition of wall expansion during long-term chilling

5.6 Genotypic variation in thermal responses of wall extensibility **124**
5.6.1 The example of *slender* barley
5.6.2 The importance of leaf extension zone length
5.6.3 Thermal control of wall extensibility: a model

5.7 Final conclusion and future research **130**

List of abbreviations and terminology

A	Membrane surface area	m^2
C.I.	Confidence Interval	
DD	Degree days: number of days times mean temperature above T_b	$^oC\ d^{-1}$ above T_b
DD_{emerge}	The number of degree days required for shoot emergence	DD
L	Volumetric hydraulic conductance, for a single cell it is equal to $Lp.\ A/V$. Also applies to average conductance of tissue through which water travels to reach cell.	$MPa^{-1}\ s^{-1}$
Lp	Hydraulic conductivity	$m\ s^{-1}\ MPa^{-1}$
LAI	Leaf Area Index	
LT_{50}	Lethal temperature at which 50% of shoots are killed	^oC
LER	Leaf Extension Rate	$mm\ h^{-1}$
LEZ	Leaf Extension Zone	mm
LVDT	Linear Variable Displacement Transformer	
MC	Mechanical Compliance	$mm\ g^{-1}$ or $\%\ g^{-1}$
PPFD	Photosynthetic Photon Flux Density	$\mu mol\ m^2\ s^-$
Q_{10}	The increase in the rate of a response for a 10°C increase in temperature	
RUE	Radiation Use Efficiency	Dry matter MJ^{-1} PPFD
RH	Relative Humidity	%
s.e.	Standard error	
T_b	Base temperature, temperature at which a process ceases.	^oC
WLF	Wall Loosening Factor	
XET	Enzyme with Xylogucan endotransglycosylase activity	
Y	Yield threshold of the wall, below which no extension will occur.	MPa
V	Cell volume	m^3
Ψ_p	Turgor pressure	MPa
Ψ_s	Osmotic potential	MPa
ϕ	Wall extensibility, the rate of irreversible flow of the wall for a given stress. Measured as the slope of leaf extension by turgor pressure in intact plants.	$mm.mm^{-2}\ MPa^{-1}\ s^{-1}$

Mechanical extensibility	General term describing the capacity of wall tissue to deform irreversibly under force. Measured in killed tissue by Instron analysis, creep analysis, stress relaxation or by other novel methods.	
Mechanical deformation	Total deformation of wall tissue under constant force. (In living tissue, the component of deformation due to continued growth is removed).	mm
Mechanical Compliance	The linear slope of mechanical deformation by force applied.	mm g^{-1} or % g^{-1}
Instron plasticity	Irreversible deformation of wall tissue for a given stress. Measured in killed tissue using Instron analysis.	% g^{-1}
In vivo extensibility	Wall extensibility measure as the slope of leaf extension by externally applied force.	mm.mm g^{-1} h^{-1}
Creep	Rate of irreversible extension of tissue under constant force. Traditionally measured in killed tissue	mm h^{-1}
Biochemical Creep	Rate of irreversible extension of tissue under constant force. Measured in freeze/thawed tissue that maintains protein activity.	mm h^{-1}

Chapter 1: An Introduction to temperature and plant growth

1.1 Temperature and plant growth

As with any organisation of matter, plant growth is ultimately dependent on energy processing. For plants, the energy is provided by solar radiation but its capture and utilisation must take place within the constraints of the thermal environment. Plants spend much of their time in conditions where photosynthetically active radiation is available in excess but tissue temperatures are suboptimal. In such situations temperature will have a controlling influence over many metabolic and physical processes in the plant and may ultimately determine plant productivity.

The rate of each physiological process exhibits a thermal response with a minimum, optimum, and maximum temperature (Ong and Baker, 1985). The thermal response varies from plant to plant, depending partly on developmental history, partly on genotype. Plant species can be classified into two groups depending on the temperature range within which injury is seen to occur: Chilling sensitive plants suffer injury at positive temperatures, usually below 10-15 ^{o}C; while chilling resistant plants are only damaged by exposure to temperature below 0 ^{o}C (Levitt, 1980). In this study we are mostly concerned with temperatures above freezing. However, as these categories generally reflect the species evolutionary history, they tend to represent a shift upwards or downwards in the plants thermal range so can equally be used to classify the response to suboptimal non-injurious temperatures.

1.1.1 Temperature and growth limiting processes

Comparing the thermal responses of different processes can be useful in determining which processes limit growth at a given temperature. The thermal responses for various physiological processes in *Lolium temulentum* and *Zea mays* are given in Table 1.1. *L. temulentum* is an example of a chilling resistant grass adapted to continue physiological functions at low positive temperatures typical of temperate regions (Pollock *et al.*, 1983). In contrast the maize genotypes described in Table 1.1 are adapted to warm sub-tropical regions and can be classed as chilling sensitive. Table 1.1 also deals with the influence of phenotypic acclimation on the thermal response by making a distinction between long and short-term chilling. Controlled environment studies of suboptimal temperature effects

Table 1.1 A comparison of relative thermal response (percentage of rate at 20° C), and Q_{10} values, in a chilling resistant (*Lolium temulentum*) and a chilling sensitive (*Zea mays*) grass, following both long and short-term cooling periods.

Species	Duration*	Process	Temperature (°C)					Q_{10}	Q_{10}	slope	Reference
			2	5	10	15	20	10-20 °C	5-20 °C		
L. temulentum											
	Long-term	Relative Growth Rate	22	43			100		1.6	Linear	Pollock *et al.*, (1983)
	Long-term	Net Photosynthesis	17	31			100		2.2	Linear	Pollock *et al.*, (1983)
	Short -term	Net Photosynthesis	11	18			100		3.7	exp	Pollock *et al.*, (1983)
	Long-term	LER	4	22			100		8.5	linear	Thomas, (1983)
	Short -term	LER	0	5	24	34	100	4.3	13.3	exp	Thomas *et al.*, (1989)**
Z. mays											
	Long-term	Relative Growth Rate		0	24	59	100	10	4.1	Linear	Miedema, (1987)
	Long-term	Net photosynthesis			58	83	100	1.7		Linear	Miedema, (1982)***
	Short -term	Net Photosynthesis			33	70	100	3.1	3.7	Linear	Haldimann *et al.*, (1996)
	Long-term	LER		0	13	46	100	17.7	7.5	exp	Miedema, (1982)
	Short-term	LER		0		79	100			linear	Kleinendorst and Brouwer, (1972)

*Seedlings were grown at 20°C then transferred to suboptimal temperature for at least 7d (long-term) or for less than 2h before measurement (short-term)
**The leaf extension zone alone was cooled in this case
***Seedlings were pre-treated with a range of temperatures (5-15 °C) and photosynthesis is measured over 1 day

generally take one of two forms. In long-term chilling, plants are grown for long periods (more than 7 days) at low temperature and analysed at that temperature. Whereas in short-term chilling, plants are grown at near optimal temperatures then transferred to a lower temperature where they are analysed after a relatively short period (less than 2 h). Of these the latter probably has more relevance to plants growing in temperate climates where leaf temperatures will fluctuate widely around the daily average (Peacock, 1975a), but the observed responses may be exaggerated as no acclimation to low temperature has occurred.

The shape of the thermal response varies from plant to plant and from process to process. In general, below optimum there is a linear response down to 10-15 °C, followed by an exponential decline to the base temperature (Ong and Baker, 1985). Often the response is best described as sigmoidal, but exponential responses are also common (Miedema, 1982; Fitter and Hay, 2001). Thermal responses can be quantified directly in terms of the Q_{10} of the response *i.e.* the increase in the rate of a response for a 10 °C increase in temperature, or indirectly using degree days to measure the accumulated heat required to produce each physiological event. As the thermal responses seen in whole plants are often non-linear and can vary markedly depending on pre-treatment, the usefulness of the Q_{10} coefficient is limited (Pollock *et al.*, 1993). Some of these limitations can be overcome by using non-linear models, however this makes it difficult to compare between studies. In Table 1.1 the rates are given relative to that at the optimum to aid comparison. The responses are also categorised as either linear or exponential depending on the best-fit model. The models are limited to these two equations to highlight those processes that are more sensitive to temperature change.

In the chilling resistant *L. temulentum* the response of growth rate (relative increase in dry weight day^{-1}) to chilling appears linear, though the reduction is more pronounced in the lower temperature range. In this case growth is measured over two plastochrons (Pollock *et al.*, 1983), the plants having spent at least 30 days at each temperature (Thomas, 1983). The authors do not mention any adjustment in growth rate during this period. An approximately linear relationship between relative growth rate and temperature has been reported for many species between 5-20 °C (Grace, 1988). Ultimately the decrease in growth must result from inhibition of photosynthesis or of leaf expansion both of which will influence carbon uptake over time.

Net photosynthesis in these plants also decreases linearly with temperature with no indication of an increase inhibition in the lower temperature range. Linearity in the photosynthetic response to temperature applies within a restricted range (typically 5-20 °C), in many species from cold and warm climates (Berry and Bjorkman, 1980; Grace, 1988). Over a wider temperature range photosynthetic response can be represented by a bell-shaped curve, as increases in respiration counter increases in gross photosynthesis above the optimum (see Figure 1.1). For *L. temulentum* grown at 20°C and subject to chilling short-term, photosynthetic rates are more temperature sensitive, resulting in an exponential fall with temperature and lower rates throughout (Table. 1.1). This shows the degree of acclimation that has occurred over two weeks in the long–term chilled plants. The ability to shift the thermal range for photosynthesis is often seen in plants native to areas where temperatures are variable (Berry and Bjorkman, 1980; Baker *et al.,* 1988; Fitter and Hay, 2002).

Leaf extension rate (LER) follows the same pattern as biomass growth but with a considerably higher Q_{10} value (Table 1.1). Plants transferred to 5 °C do not show significant acclimation over 20 days, however those transferred to 2 °C appear to adjust LER upwards over the first 3 days (Thomas and Stoddart, 1984). Leaves emerging at low temperature do not show improved LER compared with those transferred after leaves have emerged (Thomas, 1983). Rapid cooling of the same species over a period of 90 minutes produces a more extreme response with LER decreasing exponentially, resulting in considerably lower LER at 5 °C (Table 1.1). This may indicate that some acclimation has occurred early on during cooling. Direct testing of acclimation of LER to low temperature in general appears lacking in the literature, although further examples are discussed in Chapter 5, section 5.2.4.

In the chilling sensitive maize the thermal range has generally shifted upwards. This is commonly seen in plants adapted to warmer climates, and is true of both photosynthesis (Berry and Bjorkman, 1980) and LER (Korner and Woodward, 1987; Woodward and Friend, 1989). The response of LER to short-term chilling appears to be an exception to the above pattern, with maize being less sensitive than *L. temulentum*. Maize also differs from the temperate species in that prolonged exposure to temperatures below 10 °C further reduces LER and eventually kills the meristem (Miedema, 1982). Tissue damage following exposure to chilling or freezing temperatures represents a distinct temperature

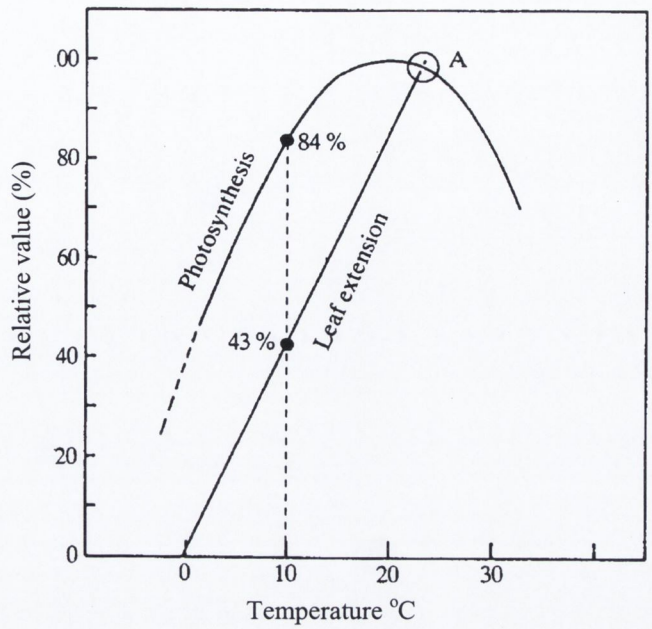


Figure 1.1 Temperature response of photosynthesis and Leaf elongation in *P. alpina* ssp. *vivipara* growing in its native habitat at c 3000 m. Point A indicates the coincidence of maximum rates of photosynthesis and elongation observed in the field. At 10 °C Photosynthesis is at 84 % and leaf extension at 43% of the maximum rate. From Korner and Larcher (1988).

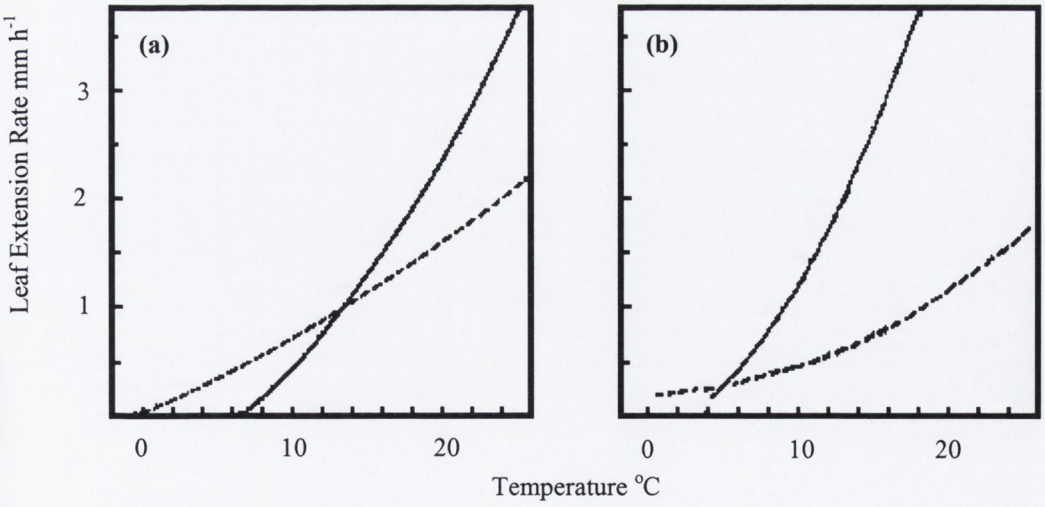


Figure 1.2 Temperature response of leaf extension in *Poa* species: (a) *P. alpina* ssp. *vivipara* (----) growing in its native habitat at *c.* 3000 m, and *P. annua* (—) growing in its native habitat at *c.* 600 m; (b) *P. alpina* ssp. *vivipara* (----) and *P. alpina* (——) growing together at mid altitude *c.* 1700. Adapted from Korner and Larcher (1988) and Woodward (1988).

limitation on growth, which is not the primary focus of this study (for an outline of such effects see Box 1.1: Chilling and freezing damage in plants).

It appears that the chilling sensitive species is less sensitive to short-term chilling than the chilling resistant species, with long-term growth below 10 °C limited neither by LER or carbon accumulation, but by damage to the tissue over time (Miedema, 1987). The chilling resistant species on the other hand, appears more sensitive to short-term chilling and maintains growth long-term by adjusting LER and photosynthetic rate, thus allowing carbon accumulation to continue at a lower temperature.

The thermal response of leaf extension differs substantially in cold adapted species. Rather than a lowering of the optimum temperature, there is a change in the slope such that plants growing in cold climates are less sensitive to temperature change even within their native range (Figure 1.2a). This adaptation allows plants to continue being active at near zero temperatures but has the consequence that when such plants are grown at warmer temperatures their ability to increase LER is restricted so they maintain relatively low rates throughout (Figure 1.2b).

In both the chilling sensitive and chilling resistant species, LER appears to be more inhibited than photosynthesis at suboptimal temperatures, indicating that it is reduced leaf extension rather than reduced carbon uptake that limits whole plant growth. This is particularly marked in the tropical species where LER decreases exponential with temperature, but is also evident in plants adapted to and growing in cold climates (Figure 1.1; Korner, 1988).

1.1.2 Carbohydrate supply at suboptimal temperatures

The evidence that photosynthesis is not limiting during chilling is supported by the observation that there is a build-up of carbohydrates in leaf tissue during cold periods (Pollock 1986; Hendry 1987; Rees *et al.*, 1988). Pollock *et al.* (1983) found that whole plant carbohydrates were significantly higher one week after transfer to cold and continued to increase over the 4 weeks of measurement. Plants held at 2 °C showed more accumulation than those held at 5 °C.

Box 1.1: Chilling and Freezing damage in plants

Chilling damage

Chilling sensitive plants such as maize, rice and tomato can be lethally damaged by temperatures above zero (usually below 10 -15 °C). The damage occurs mainly in the protoplasm as membranes undergo a transition from a liquid-crystalline to a solid gel phase; resulting in lateral separation of membrane proteins. In the short-term this results in increased permeability, reduced selectivity in membrane transport, inhibition of H^+-ATPase activity and an increase in the activation energy of membrane bound enzymes. Over time this affects whole plant processes inhibiting photosynthesis, respiration, carbohydrate translocation, and protein synthesis. Sever chilling over several days can result in solute leakage and a complete loss of cell compartmentation eventually resulting in cell death (Levitt, 1980; Taiz and Zeiger, 2002; Larcher, 2003). The effect of these changes in membrane properties on leaf extension are discussed in Chapter 5, section 3.1.

Chilling sensitive plants are more prone to such damage as their membranes have a higher percentage of saturated fatty acid chains, which causes membranes to solidify at higher temperatures. Acclimation at cool non-injurious temperatures can improve tolerance to subsequent chilling (Palta *et al.*, 1993; Somerville *et al.*, 2000; Taiz and Zeiger, 2002).

Mechanisms of freezing damage

The survival of many plants in nature and in agronomic situations relies on an ability to withstand temperatures below zero. The processes through which ice formation damages plant tissues has been well studied (see reviews by Guy, 1990; Thomashow, 1999; Thomashow, 2001; Taiz and Zeiger, 2002; Larcher, 2003), and can be divided into three categories:

- Intracellular ice formation: In unacclimated (unhardened) plants where the protoplast has a relatively high water content, ice crystals can form suddenly inside the cells; destroying the cell membranes through laceration (larcher, 2003).

- Intercellular ice formation: This occurs at less sever temperatures, just below zero, as free water in the vascular bundles and intercellular spaces quickly freezes. This in turn can result in ice forming between the cell wall and the protoplast (extracellular ice formation). When this occurs the protoplast is damaged indirectly by desiccation, as water is drawn away from the cell into the ice crystals. The lower the temperature the more water is drawn from the protoplast. As with water stress the reduced water content results in an increased concentration of salt ions and organic acids which can have toxic effects, inhibiting enzyme activity. If the temperature drops further the fine structure of the protoplast can itself be destroyed resulting in irreversible damage to the cell (Thomashow, 1999; larcher, 2003).

- Plasmolysis: In extreme cases the shrinking of the cell through desiccation can result in plasmolysis and an associated shrinking of the plasma membrane (through endocytotic vasiculation). This produces secondary cell damage as when the ice thaws the cell is unable to re-expand and bursts under the force of the returning water (Somerville *et al.*, 2000).

Mechanisms of freezing tolerance

The degree to which a plant will suffer these injuries varies between individuals depending on genotypic characteristics and on development history. Chilling sensitive species are characteristically unable to withstand any temperature below zero, for less sensitive species a period of acclimation (hardening) is often vital to allow tolerance to develop (Levitt, 1980). The mechanisms which plants use to minimise damage from frost can be broadly divided between those used to prevent ice formation from occurring and those used to minimise damage once ice formation has occurred.

Ice formation within the cells can be avoided by:

- Freeze point depression: Holding a high concentration of solutes in the cell sap (lowering ψ_s) reduces the temperature at which ice will form. This provides moderate protection from sub-zero temperatures down to about -5 °C (larcher, 2003).

- Super cooling: Some cells are able to inhibit or delay the formation of ice at temperatures below the theoretical freezing point. This is achieved using nucleation barriers such as rigid cell walls, or antifreeze proteins (Bray *et al.*, 2000; larcher, 2003; Griffith and Yaish, 2004). Antifreeze proteins have been shown to form oligomeric complexes with large surface areas; these appear to interact with ice inhibiting its expansion and recrystallization (Bray *et al.*, 2000).

Where ice formation is unavoidable, plants can minimise the damage by:

- Translocation ice formation: Moving water into intercellular spaces or frost resistant tissues increases the solute concentration in the most vulnerable cells so reducing their freezing point. (larcher, 2003).

- Accumulating compatible solutes: Compatible solutes (also termed Osmoprotectants) are highly soluble compounds that carry no net charge at physiological pH and are non-toxic at high concentrations (McNeil *et al.*, 1999). These solutes serve to lower ψ_s in the cytoplasm reducing water loss to forming ice. They also serve to stabilise proteins and membranes against toxic effects from high concentrations of salt ions and organic acids, and in some cases offer protection against low temperature itself (McNeil *et al.*, 1999; Bray *et al.*, 2000; larcher, 2003).

- Altering membrane properties: Increasing incorporation of cold-stable phospholipids and other cryo-protective compounds can stabilise membranes against freezing (Thomashow, 1999; Thomashow, 2001; Taiz and Zeiger, 2002). In some cases incorporation of phospholipids also prevents damage from plasmolysis by facilitating the formation of exocytotic extrusions, which maintain the membranes integrity and allow the cell to re-expand when the ice thaws (Somerville *et al.*, 2000).

Acclimation to freezing

Prehardening at above zero temperatures involves the accumulation of compatible solutes and other protective substances. This allows freezing point depression by lowering ψ_s, and also offers protection from freezing induced desiccation (McNeil *et al.*,1999; Thomashow, 2001; larcher, 2003). Typically the protection takes several days to develop fully, and is lost when plants are returned to warmer temperatures (Guy, 1990; Taiz and Zeiger, 2002). Several lines of evidence have shown that abscisic acid is involved with some aspects of cold acclimation, although full hardening is not always mimicked by endogenous application of abscisic acid (Taiz and Zeiger, 2002).

Additional hardening can take place when plants are subject to moderate sub-zero temperatures (0 to –5 °C), this involves changes to the membrane properties (larcher, 2003). In particular, some plants initiate an increase in unsaturated phospolipids in the plasma membrane to prevent damage from thawing ice (Somerville *et al.*, 2000).

Having said this, it is interesting to note that maintenance of high water soluble carbohydrate content during winter has been associated with freezing tolerance in a range of species (Humphreys, 1989). As such, the build-up of carbohydrate during cold periods could represent an acclimation response resulting in reduced partitioning to cell expansion rather than an incidental build-up caused by a reduction in the sink strength of the expanding regions. In addition, some of the build-up in leaf tissue could result from reduced sink strength in the root system. Substantial reductions in the demand for carbohydrates have been reported during root cooling (Farrar, 1988). Nonetheless, this observation does appear to discount the simple concept that cooling reduces leaf growth by reducing carbohydrate supply.

1.1.3 Translocation of carbon at suboptimal temperatures

Translocation in the phloem is reduced at low temperature due at least in part, to increased sap viscosity (Farrar, 1988). Kleinendorst and Brouwer (1972) found that cooling of the leaf above the meristem to 5 °C resulted in a temporary drop in translocation of carbon to the meristem and reduced LER. However, the inhibition of LER was temporary and was not as great as when the meristem itself was cooled, indicating that translocation is not a limiting factor when the whole plant is cooled.

Crawford and Huxter (1977) showed that for maize roots grown at 2 °C, reduced respiration and growth rates were associated with lower sugar content in the expanding tip. They also found that both respiration and tissue expansion were stimulated when glucose was exogenous applied. This would suggest a role for carbohydrates in controlling expansion, however the recovery was only slight and no effect was seen for roots growing at 6 °C. For maize, prolonged treatment with 2 °C would be expected to cause injury, thus the reduced carbon transport reported in this case may result from damage to the translocation pathway. Translocation does not appear to be as temperature sensitive in chilling resistant plants where whole leaf cooling results in an accumulation of carbohydrate in the meristematic zone (Pollock and Eagles, 1988).

Overall, translocation may limit growth in particular situations (i.e. localised cooling, rapid cooling, severe cooling) but is unlikely to be a controlling factoring during normal conditions in the field. Also, responses seen in upland (cold adapted) species, with

translocation in the light seeming to be kept artificially low at 7 °C while night time rates are actually enhanced (compared to 19 °C), suggests complex physiological responses are involved during long-term cooling (Farrar, 1988).

In general it appears that the production of dry matter at suboptimal temperatures is restricted not by carbon supply but by the process of tissue expansion itself (Miedema, 1982; Korner and Larcher, 1988; Pollock, 1993). Productivity over the growing season will be greatly influenced by the rate at which leaves grow to intercept radiation (Biscoe and Gallagher, 1977; Potter and Jones 1977), and even small reductions in LER during cooling translate into large differences in leaf area over time (Collins and Jones, 1988). As such, the thermal response of leaf elongation can be more important in determining productivity than the photosynthetic response (Grace, 1988).

1.2 Temperature and leaf extension

Having established the importance of leaf expansion in determining productivity at suboptimal temperatures, researchers have sought to identify the point of limitation in the expanding leaf. In monocotyledonous plants cell division and expansion takes place at the base of the leaf in the leaf extension zone (LEZ) (Kemp, 1980). Early experiments with grass species established that LER was more dependent on LEZ temperature than air temperature (Kleinedorst and Brouwer, 1970; Watts, 1972b; Peacock, 1975b). Watts, (1972) found that, when LEZ temperature is isolated from that of the rest of the plant, varying root or air temperature has only a minor effect on LER. The short-term effects of imbalances between root and shoot temperature (Kleinedorst and Brouwer, 1970; Watts, 1972b; Barlow, 1977; Pardossi, 1994), and temperature driven changes in evaporation (Ben-Haj-Salah and Tardieu, 1996; Clifton-Brown and Jones, 1999) add complexity to this picture (see section 5.3). Nonetheless in cases where the whole plant is cooled the LEZ appears to be the point of limitation.

Cooling of the meristem region results in a reduced LER, which can be detected within 20 s (Stoddart *et al.*, 1986). A response over this time scale cannot be due to a limitation in the supply of unexpanded cells from the meristem. Such a rapid response also makes it unlikely that long distance signalling is involved in the immediate thermal response (Pollock, *et al.*, 1993). Thus by a process of elimination the focus turned to cell expansion itself.

1.2.1 The process of cell expansion

Before reaching maturation a barley epidermal cell may expand its volume by more than 20,000 times. This is accomplished through diffuse growth of the cell walls during which the sidewalls, where most of the expansion occurs, may increase from 0.0002 to 1.6 mm in length (Schunmann *et al.*, 1997). This process occurs without weakening the cell wall and without loss of cell turgor pressure. The physical events that allow this remarkable feat have been reviewed by several authors including Cleland (1977), Taiz (1984), Tomos (1985), Steudle (1985), Cosgrove (1986), Cosgrove (1993b), and can be summarised as a cycle of events as follows:

1. Turgor pressure creates a tensile stress in the load bearing parts of the cell wall.
2. The wall loosens reducing the stress, without significantly changing cell volume (a process sometimes termed stress relaxation).
3. Turgor pressure is reduced, reducing the cell's water potential.
4. The disequilibrium in water potential drives water entry, increasing cell volume by stretching the wall.
5. To fully restore turgor pressure more solutes must be taken into the cell to compensate for the extra water in the now larger vacuole.

In an expanding cell these steps effectively occur simultaneously so that wall stress and turgor remain steady (Cosgrove, 1993b). Partitioning the process allows us to separate expansion into a number of measurable physical parameters.

1.2.2 The Lockhart equation

Lockhart (1965), formulated a model of plant cell expansion that brings together the concurrent processes of water uptake and wall yielding, and provides a mechanistic basis for analysing each process.

The role of water uptake in cell expansion is given by:

(1.2) $\mathrm{RER} = L(\sigma \,.\, \Delta\Psi_s - \Psi_p)$

For an isolated single cell Relative Expansion Rate (RER) is the relative rate of volume expansion, L is hydraulic conductance of the cell membrane (MPa^{-1} s^{-1}), $\Delta\Psi_s$ is the difference in osmotic potential between the cell and the external environment (MPa) and Ψ_p is the turgor pressure (MPa). $\Delta\Psi_s$ may be modified by σ, the solute reflection coefficient, but σ is usually assumed to be 1.0 (Taiz, 1984; Steudle, 1985). For an isolated single cell hydraulic conductance is determined solely by the membrane conductivity L*p* (m MPa^{-1} s^{-1}). In whole plants L refers to the average conductance of the water transport pathway and incorporates the volume and area geometries of the tissue (Cosgrove, 1981). In this case it is the conductance of the pathway to the nearest xylem cell that is important. Where water potential in the xylem is not measured the conductance of the whole water transport system must be considered (Cosgrove, 1986; Fricke, 2002).

The role of the cell wall is given by:

(1.2) $RER = \phi(\Psi_p - Y)$

where ϕ the rate of irreversible flow of the cell wall under stress i.e. extensibility (mm . mm^{-2} MPa^{-1} s^{-1}) and Y is the yield threshold for the cell wall below which no extension will occur (MPa).

The dual role of water uptake and cell wall extensibility are explicitly expressed by:

(1.3) $RER = [\phi . (L/ \phi + L)] . (\sigma . \Delta\Psi_s - Y)$

In this formulation it is important to note that turgor pressure does not appear explicitly. As highlighted by Lockhart (1965), turgor pressure is not an independent variable but is a function of hydraulic conductance, extensibility, and osmotic potential. In this case turgor is a function of the L / ϕ ratio. If $L << \phi$ (*i.e.* hydraulic conductance is limiting) the turgor pressure of the cell will approach the yield threshold and equation (1.3) will reduce to equation (1.1) (Cosgrove, 1981, Tomos, 1985, Cosgrove 1993b). When L is not limiting equation (1.3) reduces to equation (1.2). When L and ϕ are comparable in size and expansion is co-limited by hydraulic properties and wall yielding (Cosgrove, 1981; Fricke, 2002), again equation (1.2) should hold.

Equation (1.2) has been validated through experiments with giant cell alga (Taiz, 1981; Tomos, 1985) and later in higher plants (Tomos, 1985; Pritchard, 1994; Fricke, 2002). However, experiments suggest that the wall properties ϕ and Y are not fixed over time and must be viewed as variables; changing as a plant develops and in response to environmental conditions (Tomos, 1985).

In physical terms, equation (1.2) identifies two possible control points that can determine LER: turgor pressure and cell wall properties. The role of each parameter in modulating expansion rate is discussed below. The influence of temperature on each of these parameters is examined in Chapter 3.

1.2.3 Cell expansion and turgor pressure

In expanding plant cell turgor pressure typically ranges from 0.3-1.0 MPa, depending on species and development history (Cosgrove, 1993a). Turgor of individual cells does not appear to change during expansion despite large changes in expansion rate through their lifespan. This has been shown in the root extension zones of several species (Spollen *et al.*, 1993; Pritchard, 1994), and indicates that turgor does not regulate cell expansion rates under non-stressed conditions. Similarly turgor pressure does not appear to be a primary factor in regulating genotypic differences in LER (Table 1.2).

Under stressed conditions, variation in turgor pressure has a clear effect on LER, particularly during the early stages of water deficit (Spollen, *et al.*, 1993). Several studies have correlated changes in LER with changes in the turgor pressure of expanding cells; measured directly using the pressure probe (Shackel *et al.*, 1987; Pardossi *et al.*, 1994), or estimated by the difference between water potential and cell osmotic potential (Taylor and Davies, 1986; Cramer and Bowman, 1991; Pardossi *et al.*, 1994). Such correlations have been seen when water supply is limited, during drought (Matthews *et al.*, 1984), salt stress (Cramer and Bowman, 1991), or root cooling (Pardossi *et al.*, 1994); or when transpiration load is altered by manipulation of Vapour Pressure Deficit (VPD) (Taylor and Davies, 1986), or by leaf shading (Shackel *et al.*, 1987). Cooling has the potential to reduce turgor pressure by limiting water supply, through effects on hydraulic conductance and/or the maintenance of osmotic potential in the expanding cells. These aspects are discussed in Chapter 3.

Table 1.2 The relationship between leaf extension rate (LER) and turgor pressure of the expanding cells in various barley genotypes. Turgor pressure was measured using the pressure probe, or estimated by subtracting leaf extension zone osmotic potential from xylem water potential (*).

Genotype	LER ($\mu m\ min^{-1}$)	Turgor (MPa)	Reference
H. vulgare L. cv. Herta (wildtype)	11.2 ± 3.2	0.65 ± 0.07	Pollock *et al.* (1990)
H. vulgare L. cv. Herta (*slender*)	29 ± 3.8	0.69 ± 0.05	Pollock *et al.* (1990)
H. vulgare L. cv. Harrington	30	0.24 ± 0.03*	Cramer (2003)
H. vulgare L. cv. Golf	34	0.56 - 0.57	Fricke (2002)
H. vulgare L. cv. CM72	45	0.30 ± 0.1*	Cramer (2003)

1.2.4 Cell expansion and cell wall properties

Clearly, the extensibility of the cell wall (ϕ) has the potential to limit cell expansion. Of all the physical steps contributing to expansion, the loosening of the wall in response to tensile stress is the only one missing in non-expanding cells. The mechanism of wall loosening is not yet fully understood (Cosgrove, 2002), but analysis of wall composition does provide some important clues.

The fundamental framework of the wall is constructed of cellulose microfibrils and cross-linking glycans embedded in a hydrated matrix of pectic polysaccharides (see Box 1.2: The expanding cell wall; Carpita, 2002; Cosgrove, 2002). Cellulose microfibrils are effectively inextensible but cell expansion may occur as the binds between cellulose microfibrils are loosened, causing the network to reorganise in a way that increases the surface area (Cosgrove, 2002). Precisely how the load bearing bonds are organised is not yet known, but it is likely that cross-linking glycans that bind to cellulose microfibrils play a key role (see Figure 1.3; Cosgrove, 1999).

There are many cases where chemical and environmental changes can be seen to affect both wall properties and LER. To correctly interpret such correlations it is necessary to look at the methods used to measure wall properties.

Much of the early work on cell expansion focused on the mechanical properties of isolated cell walls (see reviews by Cleland, 1971; Sellen, 1980; Taiz 1984; Cosgrove, 1993a). This was partly an attempt to ascertain information on the molecular arrangement of the cell wall, and partly due to a belief that cell expansion may be a purely physical process, in which case mechanical properties would be all important in governing the rate of extension *in vivo*.

Three principle methods have been used to assay cell wall mechanical properties: creep analysis (constant force), stress relaxation (constant strain), and Instron analysis (constant strain rate). These methods are described in Box 1.3: Measuring cell wall rheology.

Before carrying out these assays the influence of turgor pressure is removed by subjecting the isolated walls to a freeze/thaw cycle. Traditionally, isolated walls are also heat treated to remove the undefined effects of wall enzymes (Taiz, 1984). These wall preparations

Box 1.2: The growing cell wall

The cell wall maintains the structural integrity of the cell but is also metabolically active and undergoes compositional and conformational changes during cell development, which influence the rate and direction of growth (see reviews, Showalter, 1993, Brett and Waldron, 1996; Caprita, 1996; Cosgrove, 1999; Carpita and McCann, 2000; Mc Cann, 2001; Cosgrove, 2002). The growing cell wall consists of a network of cellulose microfibrils and cross-linking glycans embedded in a hydrated matrix with pectins, structural and non-structural proteins (Figure 1.3). Each of these components is descried below along with an assessment of their potential role in regulating cell expansion.

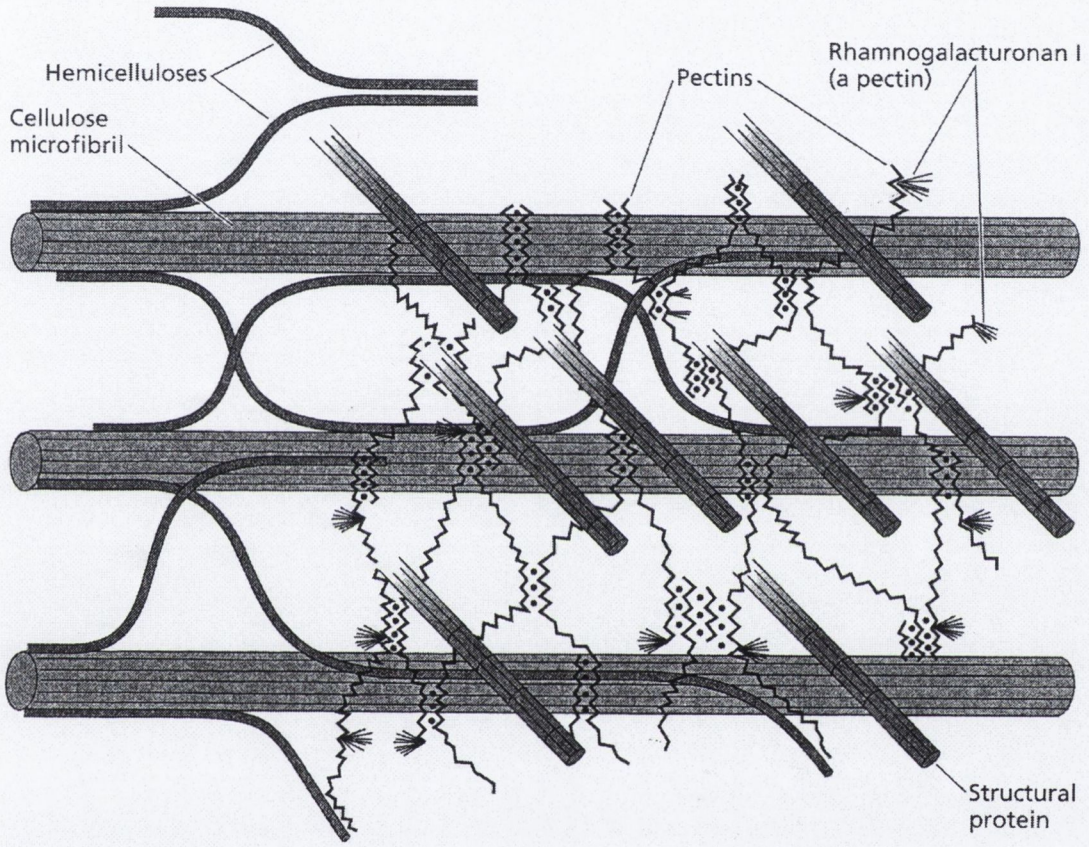


Figure 1.3 Schematic diagram of the primary cell wall. Showing the major structural components and their likely arrangement. Cellulose microfibrils are coated with hemicelluloses (cross-linking glycans), which may also crosslinks the microfibrils to one another. Pectins form an interlocking matrix gel, perhaps interacting with structural proteins. (from Brett and Waldron, 1996; Cosgrove 2002).

Cellulose microfibrils

Cellulose microfibrils are strong structures relatively resistant to enzyme attack, which provide the structural frame of the cell wall. According to current models of cell extension microfibrils neither stretch nor break during expansion, rather they slip apart from each other following the reorganisation of other matrix polymers that hold them in place (Cosgrove, 2000a). The orientation of microfibril is crucial in determining the nature of expansion. For directional expansion the internal load-bearing microfibrils must be arranged perpendicular to the axis of expansion. In most cases they are arranged transversely in the lateral walls facilitating longitudinal expansion (Cosgrove, 2002).

Cross-linking glycans

The cross-linking glycans bind noncovalently to the surface of cellulose, perhaps forming tethers that bind cellulose microfibrils together, or acting as a lubricating coating to prevent direct microfibril-microfibril contact (Cosgrove, 2000a). Two major cross-linking glycans are found in plants; xyloglucans are found in all dicots and most monocots, while glucuronoarbinoxylans are the major group in the grasses and other commelinoids species. A third major cross-linking glycan (1→3),(1→4)-β-D-glucan (*β*-glucans), is found only in grasses (Carpita and McCann, 2000). The relative amount of each cross-linking glycan changes with developmental stage and can vary between cell type and within a cell. Theses changes may have a role in regulating developmental processes including cell expansion, but their precise functions are not known (Carpita and McCann, 2000; Cosgrove, 2000).

Pectins

Pectins together with other wall constituents form the interlocking hydrated matrix in which the network of cellulose and cross-linking glycans is embedded. Pectic substances may also be involved with controlling metabolism in the wall, and could potentially regulate the activity of wall loosening enzymes by limiting wall porosity. In addition, the occurrence of certain pectins has been associated with periods of cell expansion (Carpita, 1996; Carpita and McCann, 2000).

Wall Proteins

In his review of cell wall proteins Cassab (1998) points to their perplexing diversity. In many case their function is unknown, although in most cases functions have been suggested (for review see, Showalter, 1993; Carpita, 1996: Cassab, 1998; Cosgrove, 2002). They can be divided into two categories:

Structural proteins

The majority of cell wall proteins are cross-linked into the wall and probably have structural roles. They can be categorised according to their dominate amino acids as Hydroxyproline-rich glycoprotein (HRGP), Proline-rich protein (PRP) or Glycine-rich protein (GRP) (Cassab, 1998; Showalter, 1993). Extensin a HRGP, is the most well-studied cell wall structural protein. It was originally thought that it may play a role in cell expansion, but it has since been shown that formation of the extensin-cellulose network increases the tensile strength of the wall. The peak in extensin levels is associated with the cessation of growth perhaps having a role in fixing the final shape (Cassab, 1998, Cosgrove 2002). In grasses structural proteins are less common and their function seems to be filled by phenolic substances (Caprtita, 1996).

Non-structural Proteins

Cell walls also contain proteins that are not linked to the structural matrix but function to modify the wall polymers at various stages of development. As with the structural proteins they are a diverse group and are thought to be involved in regulating various processes, from wall modification to metabolite transport and cell signalling (Cosgrove, 1993, Cosgrove, 1997). Several of these proteins are though to be involved in modifying wall architecture during cell expansion (see review: Fry, 1995; Cosgrove, 1997; Cosgrove 1999). Of particular interest are the hydrolases, xyloglucan endotransglycosylase (XET) and expansins. Theses are discussed in the main text.

Box 1.3 Measuring cell wall rheology

Several techniques from the material sciences have been adapted for measuring rheology in plant cell walls. Use of these techniques has been reviewed previously by Cleland (1971), Taiz (1984), Tomos (1985), Masuda (1990), and Cosgrove (1993). Below is a brief account of the three principle methods.

Wall Creep Analysis

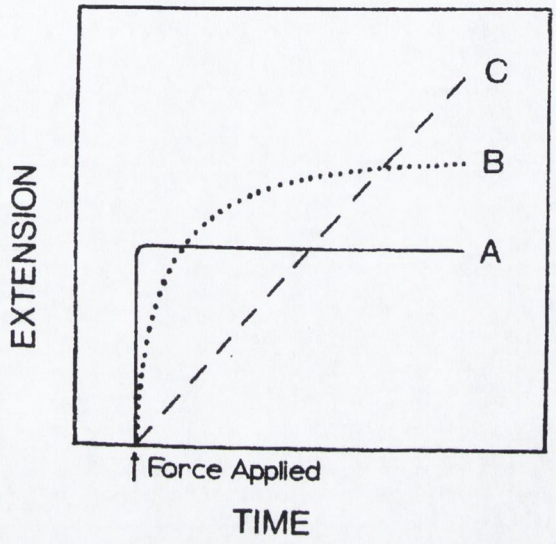


Figure 1.4 Schematic diagram of a creep assay, illustrating the relationship between extension and time for instantaneous elastic extension (A), viscoelastic extension (B) and viscous flow (C). (From Cleland, 1971).

Wall creep analysis measures the time dependent extension of a sample under constant force. The type of extension that a material undergoes depends on the number of crosslinks and the entanglements that are present in the material (Figure 1.4). At one extreme, if the material is bonded together by extensive crosslinks such as exists in rubber, extension will be instantaneous and entirely elastic (A). At the other extreme, if few crosslinks are present as in the case of oil, the extension is directly proportional to time and is irreversible i.e. viscous flow (C). In cell wall tissue the extension is an intermediate form, a viscoelastic extension typical of composite materials. When a force is applied there is a certain amount of instantaneous extension, but the extension then continues at a rate that is nearly proportional to log time (B). The latter time dependent extension is termed 'creep'. Such a response involves the breakage of bonds in response to stress, but the deformation is limited and so decays over time.

In addition to the traditional creep analyses illustrated in Figure 1.4 there are two variants commonly used with plant tissue. The first variant is an abridged version where the wall is put under constant load for a short time and then released. The irreversible and reversible changes in length are then taken as measures of plastic and elastic deformation respectively. The second variant uses the same procedure as for Figure 1.4, but the tissue tested is not heat inactivated prior to the assay so the metabolically mediated element of extension is retained. The resulting extension is here termed 'biochemical creep', and reflects both the mechanical properties of the wall and the effect of biochemical activity that loosens the wall during growth. This method is discussed further in the main text.

Instron analysis

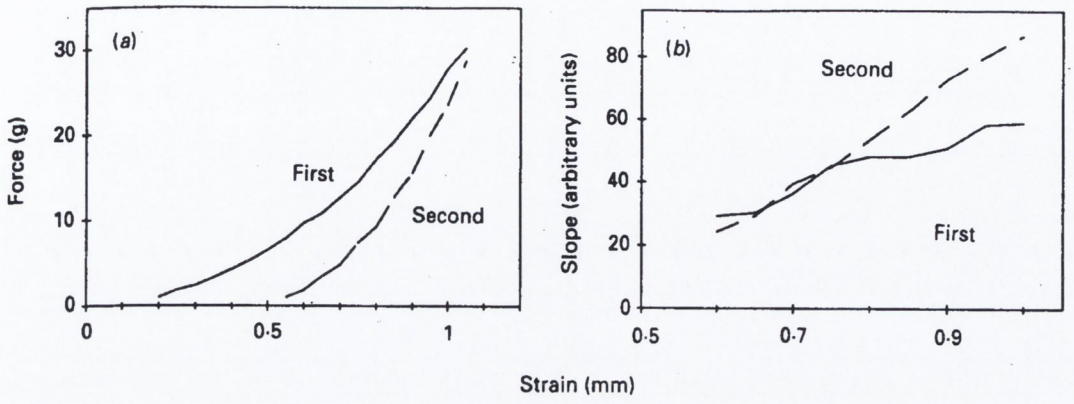


Figure 1.5 An example of output from an Instron analysis. 0.5 mm segments of tissue from cucumber hypocotyls were frozen, thawed, heat inactivated and extended at a rate of 10 mm min^{-1} in an extensiometer. (a) Force is plotted as a function of tissue extension in two consecutive extensions. (b) The slopes of the two curves continue to increase as the walls are stretched. (from Cosgrove, 1993)

Figure 1.5 shows the results of a typical Instron analysis. Wall tissue is extended at a rapid and constant rate allowing stress to build up. Tissue is extended once, then returned to its original length and extended a second time. The difference between the first and the second curve represents the plastic (irreversible) element of extension. Usually expressed as the plastic compliance, the reciprocal of the slope (stress/strain), high compliance meaning high deformability. Alternatively the parameters are simply expressed as strain/force (with no account of tissue area) and expressed as % per force, and simply called plastic extensibility or as used here 'Instron plasticity'.

Stress relaxation

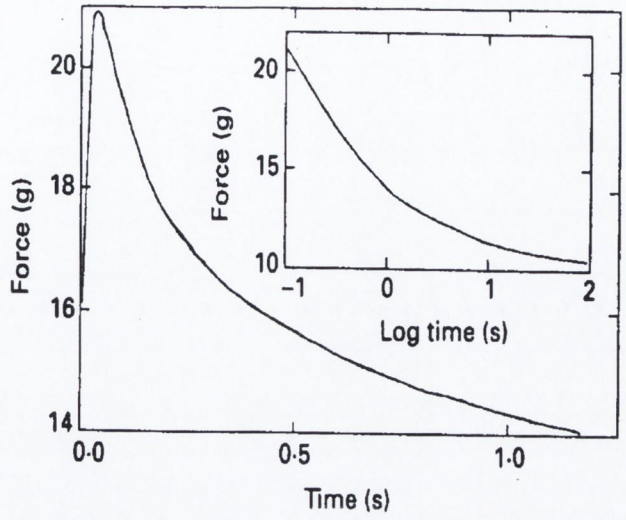


Figure 1.6 An example of output from an Stress relaxation analysis. 0.5 mm segments of tissue from cucumber hypocotyls were frozen, thawed, heat inactivate and extended rapidly in an extensiometer until a force exceeding 20g was obtained then held at a constant strain and the decay in force was recorded. The inset shows the relaxation of force against log time. (from Cosgrove, 1993)

In stress relaxation analysis tissue is rapidly extended then held at a constant length, the extension increases the stress on the sample, once stretching has stopped any loosening of the wall cause a relaxation in the stress (Figure, 1.6). This technique allows an analysis of the timescale of the relaxation, which can be divided in to several parameters providing information about bonding in the wall.

show viscoelastic properties typical of composite materials (Cosgrove, 1993a). Their extension properties are intermediate between elastic solids and viscous liquids as illustrated using the creep assay (see Box.1.3: Figure. 1.4).

Early researchers using giant cell algae and coleoptile material, soon concluded that the viscoelastic response seen in isolated walls could not account for the linear extension seen in living cells. Expansion *in vivo* is not a purely physical stretching of the wall, but requires a series of independent extension steps (Cleland, 1971). Changes in mechanical wall properties were correlated with changes in expansion rate in certain situations, such as pre-treatment of tissue with auxin, but the change in mechanical properties were not sufficient to explain the differences in expansion rate (Cleland, 1971). There is now considerable evidence that walls can change their extensibility (ϕ) without any change in their viscoelastic properties. Thus mechanical assays may be misleading if they are taken as a simple measure of expansion properties (Pritchard, 1989; Cosgrove, 1993a). Given the lack of similarity to *in vivo* cell expansion these assays cannot be taken as measures of ϕ and Y, and should only be used as indicators of differences in wall architecture that may or may not relate to differences in cell expansion rate.

Despite this, the Instron technique appears to be the most widely used method of examining wall properties, and continues to show good correlation with *in vivo* expansion in certain situations (Matthews *et al.*, 1984; Pritchard *et al.*, 1990; Soga *et al.*, 1996; Parvez *et al.*, 1996; Nakamura *et al.*, 2003).

Cleland (1971) predicted that each wall extension step must involve a biochemical modification of the wall, and that this biochemical step likely limits the rate of extension, thus explaining the lack of correlation between mechanical properties and tissue expansion. Cleland *et al.* (1987) showed this was the case when they carried out a creep analysis using living wall tissue submerged in an acidic buffer. In this case the samples were not heat-treated following the freeze/thaw cycle, so protein activity and metabolism were maintained to some degree. As with killed walls, there was an initial viscoelastic response which was largely completed after 20-30 minutes, but under acidic conditions a second phases of extension was seen. This second phase was a long-term creep that was linear with time and persisted for 6 h in *Avena* coleoptiles and up to 48 h in cucumber hypocotyls.

This long-term creep was analysed further by Cosgrove (1989), who showed creep rate was inhibited by addition of various denaturants to the acidic buffer or by boiling the tissue prior to measurement, indicating that long-term creep was enzymatically driven. He also found a good correlation between this 'biochemical creep' and extension rate in plants grown under control and red light treatments. Subsequent studies using similar assays have shown correlation between biochemical creep and expansion rate in different tissues including, air grown and submerged rice internodes (Cho and Kende, 1997); dark and light grown oat hypocotyls (Cosgrove and Li, 1993); well-watered and water stressed maize roots (Wu *et al.*, 1996); and following a spatial gradient in cell expansion in oat hypocotyls (Cosgrove and Li, 1993). Figure 1.7 shows the output of a long-term creep analysis comparing the extension of 'Native' tissue where proteins are still active, and 'Boiled' tissue which has been heat inactivated so as only the mechanical aspects of extension remain as in the traditional creep assay.

The work of Cleland *et al.* (1987) and Cosgrove (1989), confirmed the involvement of some wall loosening factor (WLF) that is present in living cell walls and suggested an enzyme with a pH dependence. A key breakthrough came in 1992 with the discovery that the long-term creep could be restored in denatured walls by adding back certain wall proteins. The active proteins used in these reconstitution assays were subsequently named expansins (Cosgrove, 2000).

1.2.5 Wall loosening factors

As described in Box 1.2 all of the components of the cell wall have been suggested to influence cell expansion in specific situations, for example, many compositional changes are associated with the rigidification of the wall during the final stages of maturation (Cosgrove, 1997). However, the regulation of cell expansion rate under normal circumstances must be under the control of agents with specific wall loosening activity. A WLF must be able to initiate the reorganisation of the wall polymers rather than just constrain the extent of the extension once wall loosening has begun.

Hydrolases

Initially, a strong contender for the mechanism of wall loosening was hydrolysis. Cell walls contain numerous enzymes capable of hydrolysing cross-linking glycans, and it was

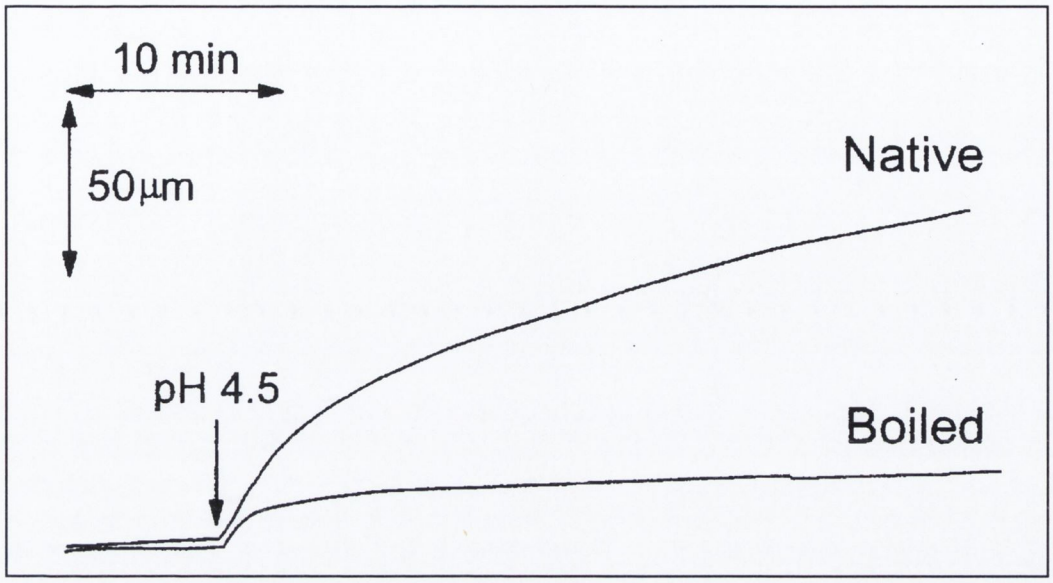


Figure 1.7 Long-term creep in elongating tissue from rice internodes. 10 mm segments of tissue were frozen, abraded, thawed and held under a constant load of 10 g in an extensiometer. After pre-treatment with 50 mM Hepes/Tris, pH 6.8 buffer, for 20 min, the solution was changed for 50 mM sodium acetate, pH 4.5, at the indicated point. Extension is shown for both Native tissue in which proteins are still active and Boiled tissue which has been heat inactivated so as only the mechanical aspects of extension remain. (From, Cho and Kende, 1997).

envisaged that these enzymes might function to loosen the wall by breaking load bearing links between cellulose microfibrils (Cosgrove, 1999).

In the case of grasses it was proposed that exo- and endo-β-D-glucanases might cleave the cross-linking glycans (*β*-glucans) through hydrolysis, releasing the cellulose microfibrils. This was particularly plausible as *β*-glucans are developmentally regulated, being absent from meristematic cells but accumulating during cell expansion and decreasing as elongation rate falls (Carpita, 1996). In addition, when antibodies against exo- and endo-β-D-glucanases were added to active walls expansion was inhibited, suggesting they have some role in extension in the grasses (Carpita and McCann, 2000).

Several hydrolytic enzymes were tested for their ability to induce extension in isolated walls using the long-term creep assay (as used in the expansin reconstitution assays). They either failed to effect wall extension or caused the wall to break without an intermediate period of wall creep; suggesting hydrolysis is not the primary mechanism of wall loosening (Cosgrove, 1999).

Some authors contend that in grasses the metabolic turnover of *β*-glucans by (1→3),(1→4)-β- D-glucanases results in changes to wall architecture that regulate expansion rate *in vivo* (Nakamura *et al.*, 2003 and references therein). However, other studies show that the occurrence of exo and endoglucanase in the wall is not correlated with cell expansion but rather with the turnover of the *β*-glucans that occurs after expansion has ceased (Carpita, 2001).

XET

A number of wall enzymes have been identified as having the ability to cut xyloglucans and join them with other free xyloglucans (Rose, 2002). This xyloglucan endotransglycosylase (XET) activity has been suggested to have a role in wall loosening (Fry *et al.,* 1992; Nishitani, 1997).

There are several reports that the highest XET activity is associated with regions of cell expansion (Fry *et al.*, 1992; Pritchard *et al.*, 1993b; Wu *et al.*, 1994; Smith *et al.*, 1996). An increase in the level of XET-related mRNA has been shown during leaf extension in *Festuca* (Reidy *et al.*, 2001) and during the promotion of soybean epicotyl extension by

brassinosteroid (Zurek and Clouse, 1994). Furthermore, Smith e*t al.* (1996) found fast growing '*slender*' barley plants contained elevated levels of XET activity and mRNA levels, especially in the expanding basal regions. However, some studies have failed to find a good correlation between XET and cell expansion, with XET activity peaking before (Palmer and Davis, 1996) or after (Fry *et al.*, 1992) the point of maximum cell expansion.

Attempts to demonstrate a direct effect of XET on cell expansion in isolated walls have not been successful with the researchers concluding the XET activity alone is neither sufficient nor necessary for long-term biochemical creep (McQueen-Mason *et al.*, 1993). This suggests that XET is not the primary loosening agent and that the correlation with cell expansion may results from a role in wall assembly (Wu and Cosgrove, 2000; Thompson and Fry, 2001). Most recently it has been suggested that XET may function as a 'secondary wall loosening agent' acting to modify the wall architecture in a way that enhances the action of the primary WLF but does not promote extension when the WLF is absent (Cosgrove, 1999; Wu and Cosgrove, 2000).

Expansins

The wall proteins expansins are the only proposed WLF which have shown the ability to stimulate long-term wall creep in isolated cell walls (Cosgrove, 1999). In addition to the effects on isolated walls, expansins extracted from cucumber hypocotyls have been shown to greatly stimulate cell expansion in cell cultures from a range of species and tissue types (Cosgrove, 1999).

Gene expression studies have shown a strong correlation between expansin activity and cell expansion rate (Cosgrove, 1999). However such correlations are not absolute. For example Caderas *et al.* (2000) found expansin levels were higher in slow growing light-treated hypocotyls than in rapidly elongating dark treated tissue. They suggest that extension is likely to be controlled by expansins acting in concert with other factors that may limit cell expansion under some physiological conditions.

Experimental evidence suggests that expansins act in a catalytic way, disrupting hydrogen bonds between cross-linking glycans and the cellulose microfibrils they hold together, so allowing the wall to yield (McQueen-Mason and Cosgrove, 1994; Cosgrove, 2000). In a

wall under tension, cross-linking glycans released from the cellulose surface could move and re-bond elsewhere. This is the current model for wall loosening and would allow a staggered extension producing the long-term linear biochemical creep, as distinct from the breakage induced by wall hydrolyses (Cosgrove, 2000; Carpita and McCann, 2000).

1.3 Conclusion

The extent to which leaf extension is reduced at suboptimal temperatures clearly has a vital role in determining plant productivity in temperate climates. Across different plants there is a range of thermal responses in leaf extension that can only be explained through an understanding of the mechanisms controlling cell expansion itself. This must incorporate the role of water entry in providing the driving force for cell enlargement, and the role of the cell wall in constraining this force whilst simultaneously allowing the cell to expand. Recent advances in our understanding of wall loosening factors provide a new paradigm in which to describe the control of cell expansion, this too must be incorporated into our model of temperature control of cell expansion.

This thesis aims to explore the mechanisms through which low temperature influences the rate of plant growth. Chapter 2 investigates the role of low temperature in mediating leaf emergence, leaf extension and seedling survival. Genotypic variation in the thermal response of each process is described and its impact on plant productivity is assessed. Chapter 3 aims to identify the point at which suboptimal temperatures act to inhibit leaf extension. It is hypothesised that reduced cell expansion at suboptimal temperatures is associated with a fall in turgor pressure and/or cell wall extensibility. Chapter 4 analyses cooling induced inhibition of cell expansion *in vivo*, and compares the thermal response of leaf extension in three contrasting plants. It is hypothesised that differences in the thermal response of leaf expansion are associated with differences in the mechanical and/or metabolic properties of the cell wall. Chapter 5, the final discussion, re-assesses results from previous studies in the context of findings from chapters 2 to 4. The different mechanisms controlling responses to short and long-term cooling are explored and a model proposed.

Chapter 2: Frost tolerance and thermal response of leaf growth in four *Miscanthus* genotypes. The impact of genotypic variation on potential yield.

2.1 Introduction

Miscanthus is a tall perennial rhizomatous grass that has been identified as having strong potential as a biomass crop (Jones and Walsh, 2001). The genus *Miscanthus* appears to have its origins in E. Asia (Greef and Deuter, 1993). It has been shown to grow well in cool temperate climates and is of particular interest because it utilises C_4 photosynthesis and so has a high radiation, nitrogen, and water use efficiency compared with most C_3 plants (Beale *et al.*, 1999; Jones and Walsh, 2001).

Productivity trials testing a number of *Miscanthus* genotypes have been established in Sweden, Denmark, England, Germany and Portugal, and have shown the potential for high yields on many sites (Clifton-Brown *et al.,* 2001). Harvested material has been shown to be suitable for combustion (Lewandowski and Kicherer, 1997; Wagenaar and Vandenheuvel, 1997; Visser and Pignaelli, 2001) and as a source of fibre for paper and board manufacture (Lewandowski, 1998b; Kjeldsen *et al.*, 1999; Cappelletto *et al.*, 2000; Visser and Pignaelli, 2001).

Although *Miscanthus* genotypes have produced high yields in many sites, further development of the crop is needed. A series of breeding programmes are underway across Europe with the aim of increasing yield. The identification of characteristics related to low temperature growth and frost tolerance have been identified as a key focus of such programmes (Jorgensen and Muhs, 2001; and references therein).

Suboptimal temperatures have been a key limitation in attempts to establish *Miscanthus* genotypes in Northern Europe perhaps because many of the genotypes used originate in subtropical areas (Hodknison, 1997; Christian and Haase, 2001). Previous research has looked at the influence of temperature on *Miscanthus* productivity primarily through its effects on leaf expansion (Clifton-Brown, 1997; Clifton-Brown and Jones, 1997) and photosynthesis (Beale, 1995; Weng and Ueng, 1997; Long and Beale, 2001). The temperatures required to initiate shoot emergence in spring will also influence productivity. Emerging earlier in the year effectively lengthens the growing season

increasing the amount of time the canopy is available to intercept solar radiation. This can be particularly important in the first year after planting when a long growing season is crucial to allow sufficient rhizomes stores to develop, improving survival during the first winter (Lewandowski *et al.*, 2000a).

In Northern Europe, early emergence will only be advantageous if the shoots produced can tolerate the short periods of frost that are typical during spring. Ziegenhagan (1995) identified losses from both spring and winter frosts as potential obstacles to the establishment of *Miscanthus* in northern Europe. We have previously looked at the variation in rhizome tolerance to winter frost (Clifton-Brown and Lewandowski, 2000). Here we examine the base temperature (T_b) for shoot emergence and the lethal temperature (LT_{50}) for shoot frost damage in four *Miscanthus* genotypes. The genotypes chosen have demonstrated the ability to produce high yields during field trials, while at the same time showing considerable variation in the response of yield to different site conditions (Clifton-Brown *et al.*, 2001). The growth conditions and treatment regimes used in theses experiments are based on ten years of climate data from a German field site and are designed to test the response of emerging shoots to the range of temperatures seen during spring. The importance of each of the traits in determining yield is explored using a productivity model run with climate data from the German site. The analysis aims to identify growth characterises of *Miscanthus* genotypes that may increase biomass yield within northern Europe or may help extend the range of *Miscanthus* into colder climates.

2.2 Materials and Methods

2.2.1 Field Site

A field trial to assess the biomass yield of several *Miscanthus* genotypes was established in Ihinger Hof research farm, University of Hohenheim (48°40'N: 09°00'E), Stuttgart, Germany in 1997 (Clifton-Brown and Lewandowski, 2002). Climate data was collected from a weather-station near the site. Temperature at the soil surface was measured on site using resistance thermometers and a datalogger (Star Log, Australia). The temperature range measured from 1988-1998 (Figure 2.1), was used when formulating the experimental design and in modelling potential yields. The growing season for *Miscanthus* genotypes begins with emergence in March/April and extends until flowering

occurs in August/September or in the case of Gig-2 (where no flowering occurs in Germany) when the first autumn frosts kill the shoots (Clifton-Brown *et al.*, 2001).

2.2.2 Plant material

Using the results of field trials with fifteen genotypes in five sites across Europe (Lewandowski, 1998a), four genotypes with apparently contrasting temperature responses were identified. The four genotypes, the characteristics of which are described in Table 2.1, were obtained from TINPLANT (Magdeburg, Germany), and have previously been tested for biomass yield and rhizome frost tolerance (Clifton-Brown and Lewandowski, 2000). Rhizome material was removed from the field site in Germany in the overwintering state and kept in cold storage prior to use.

2.2.3 Shoot Emergence

Plants were propagated from rhizome pieces (8-12 g FW) in which extra shoot initials were removed until one healthy initial remained. Ten randomly selected rhizomes from each genotype were placed in trays of coarse sand which were covered to maintain humidity and watered every second day to field capacity. The rhizomes were grown in a series of dark incubators held at 7 °C, 9 °C, 11 °C, 13 °C and 15 °C. Soil temperatures were monitored with thermocouples and recorded with a datalogger (Type 21X, Campbell Scientific, Leicestershire, UK). Temperatures remained within ±1 °C of the desired values throughout the experiment.

The shoot emergence experiment was run for 60 days, shoot height was measured every second day with a ruler. Shoot emergence day for each rhizome was taken as the measurement day on which a 1 cm increase in shoot height was recorded.

The percentage of rhizomes producing shoots at each temperature was calculated. Where less than 50% emergence occurred the treatment was excluded from further analysis. In some cases rhizomes were identified as nonviable during the experiment and were discounted from the total for that treatment. Shoot emergence rate was calculated as the reciprocal time to median (50%) emergence. Median emergence was estimated using Probit analysis, with cumulative per cent emergence at each temperature transformed to

probits and regressed against time using the Probit Analysis function in SPSS (SPSS Inc., 2001). Base temperature (T_b) was calculated as the x-intercept from plots of the reciprocal time to median emergence against temperature (Steinmaus *et al.*, 2000).

The number of degree days required for shoot emergence (DD_{emerge}) was calculated by multiplying the number of days to median emergence by the treatment temperature above T_b. Given a linear relationship between shoot emergence rate and temperature above T_b, the DD_{emerge} should be equal at all temperatures (DD_{emerge} was calculated for each temperature and a mean value used).

After emergence the length of the first leaf was monitored for 14 days. Leaf extension rate (LER) was calculated as the slope of leaf length (cm) by the time interval (days).

2.2.4 Frost tolerance

Individual plants were propagated from rhizome pieces as described above. Rhizomes were potted in 5x5x5 cm pots and grown at 20 ± 2 °C for 21 days, by which time 3-4 leaves had emerged. Prior to the frost treatment, plants were prehardened in a growth room at 12 ± 2°C for 8 d (12h photoperiod, 200 μmol m^{-2} s^{-1}PPFD). Conditions were based on typical climate conditions during the early spring as measured near the German site (mean temperature for April/May over the 10 years: 11.5 ± 3 sd).

For frost treatment, plants were transferred to a custom-made temperature control cabinet (after, Neighbour *et al.*, 1990; Clifton-Brown, 1997). Only the shoots were cooled with the below ground parts placed in a heated pipe system to prevent freezing. The frost treatments were carried out in the dark, and were achieved by reducing temperatures at 3 °C h^{-1}, until a set point was reached. Leaves were exposed to frost temperatures of -2 °C, -4 °C, -6 °C, -8 °C, and –10 °C, for 3 h. These were considered to cover the range of conditions typical in the field (minimum surface temperatures during April/May range from –2.9 °C to –8.5 °C, over the 10 years). Temperature was monitored using fine thermocouples held adjacent to the leaves. The frost treatment included a period of rewarming with the light on to simulate additional effects from photo-damage during frost periods (Figure 2.2).

After frost exposure the plants were transferred back to a growth room where they were held at 12 °C for a further 7 days. Growth was monitored by measuring the length of the first expanding leaf before treatments and again 7 days after frost treatment. Growth rate is expressed relative to the –2 °C treated plants. Shoots were deemed as 'killed' when growth was seen to have ceased. The Lethal Temperature at which 50% of the shoots were killed (LT_{50}) was estimated by Probit analysis (SPSS Inc., 2001).

The condition of the youngest 3 leaves on each plant was also scored visually before frost exposure and again 7 days later. Leaves were scored from 0-10, with 0 being totally brown and 10 being totally green. This provided an assessment of leaf area damage due to frost and associated photo-damage and may provide a method for assessing frost damage in the field.

2.2.5 Moisture content and osmotic potential

Leaf samples were taken prior to frost exposure from 6 plants of each genotype subject to the same growth conditions as described above. Samples were analysed for % moisture content following measurement of fresh weight (FW) and dry weight (DW), where:

$$\% \text{ moisture content} = 100 \times (FW-DW) / FW$$

Osmotic potential was measured by thermocouple psychrometry (HR33t, C-52 sample chamber, Wescor, Logan, UT, USA) using five replicates from each genotype. Samples were taken from the tips of the last fully expanded leaf and sap expressed from tissue after freezing in liquid nitrogen.

2.2.6 Modelling potential yield

The climate data from the German site was used in a simple productivity model as described in Clifton-Brown *et al.*, (2001). Briefly, the model calculates a daily growth increment on the basis of leaf area index (LAI), available solar radiation and radiation use efficiency (RUE). A thermal leaf area coefficient predicts LAI as a function of degree days above a threshold incorporating the influence of leaf area expansion and shoot

density. The thermal leaf area coefficient and the RUE used here are those established for *Miscanthus x giganteus* (Clifton-Brown and Jones, 1997).

Here the model is extended to predict the effect of different thermal requirements for shoot emergence and differences in frost tolerances of young shoots (see Figure 2.3). The date of emergence is determined by the T_b and the DD_{emerge}. Where the daily minimum temperature falls below the LT_{50} the LAI is reset to zero but the rate of canopy re-growth is assumed to be unaffected. In each case the model is run to predict yield on the August 20th. After this date the effect of genotypic differences in flowering time become crucial; these differences are not considered here.

2.2.7 Acknowledgement

All work was carried out in collaboration with Dr. John Clifton-Brown, Institute for Crop and Grassland Research, University of Hohenheim, Germany.

2.3 Results

2.3.1 Shoot Emergence

All of the genotypes showed a clear decrease in the percentage of rhizomes producing shoots as the incubation temperature was reduced (Table 2.2). At 7 °C three of the genotypes had a shoot production level below 10%; only Sin-H9 showed a tolerance to this temperature with 60% of the rhizomes producing a shoot.

The rate of emergence was seen to increase linearly with temperature (Figure 2.4). Genotypes Gig-2 and Sac-5 showed similar thermal response rates and T_b. In comparison the two *M. sinensis* hybrids (Sin-H6 and Sin-H9) had higher thermal response rates. Genotype Sin-H9 with a T_b of 6.4 °C emerged earliest at all temperatures. The higher thermal response rate seen in Sin-H9 is associated with a short thermal requirement for emergence (DD_{emerge}), which is significantly lower than that of Gig-2 and Sac-5 (Table 2.2).

As well as emerging earlier Sin-H9 had the highest LER at all temperatures (Figure 2.5). This appears to be due to a lower base temperature for extension, with the other genotypes exhibiting higher thermal response rates suggesting they would achieve higher rates of extension above 17 °C.

2.3.1 Shoot Frost Tolerance

Sin-H6 and Gig-2 showed a continuous reduction in growth as the temperature of the frost exposure was reduced (Figure 2.6a). Sac-5 maintained growth above –8 °C, after which growth was greatly reduced, Sin-H9 was more tolerant to this treatment with growth still above 70 %. A similar pattern was seen in the leaf condition score, with growth seeming to be the more sensitive measure of damage (Figure 2.6b).

The genotypic variation in frost tolerance and in physiological characteristic related to frost tolerance is shown in Figure 2.7a-c. There were significant differences between genotypes in the LT_{50}, Sin-H9 was the most frost tolerant with an LT_{50} of -9.3 °C. Genotypic differences in the moisture content of leaves prior to freezing appears to correlate with LT_{50}, with Sin-H9 having a significantly lower value than the other genotypes. There was no significant variation in osmotic potential between the genotypes, and no apparent relationship between osmotic potential and LT_{50} (Figure 2.7c).

2.3.3 Modelling potential yield

Overall Sin-H9 showed the best tolerance to growth at suboptimal temperatures and to late frost events, the impact of these traits was apparent in the results of the productivity model. Figure 2.8a-c shows the model predictions for length of growing season, percentage radiation interception and biomass yield of *Miscanthus*, based on the weather data from 1990-1999 at the German field site. Using the T_b , DD_{emerge} and LT_{50} estimated here for Gig-2 the model showed an average season length of 130 days with the date of emergence ranging from 12th April to 14th May depending on yearly weather conditions. Lowering the LT_{50} to that estimated for Sin-H9, made little difference to the length of the growing season or to yield as late frosts rarely occurred after 12th April. Lowering the T_b and DD_{emerge} to that estimated for Sin-H9 without changing the LT_{50}, resulted in shoots emerging on average 30 days earlier, increasing the average season length and average

yield. However, as late frost events are still common in late March/early April, canopy area was frequently reduced resulting in a large range of values for percentage radiation interception. This in turn lead to large variation in yield from year to year. Finally, lowering T_b , DD_{emerge} and LT_{50} to that estimated for Sin-H9, resulted in consistently higher yearly yields without increasing variation from year to year.

2.4 Discussion

This study has uncovered significant variation in the temperature sensitivity of *Miscanthus* genotypes. One genotype, Sin-H9 exhibited low temperature sensitivity in LER and shoot emergence rate. Shoots of Sin-H9 also had the highest frost tolerance, which was associated with significantly lower leaf moisture content in this genotype.

The response of emergence rate (reciprocal time to median emergence) to temperature change is comparable to that seen for other vegetative propagules (Holt and Orcutt, 1996), and for germination of seeds (Angus *et al.,* 1981; Roberts, 1988; Steinmaus *et al.,* 2000) with a linear relationship providing an adequate fit for all genotypes. Steinmaus *et al.* (2000) concluded that the calculation of reciprocal time to median germination provided the best estimate of base temperature (T_b) across many species with contrasting thermal responses. Evaluating the accuracy of the T_b estimate is generally done by comparing predictions with the directly observed temperature where no germination took place (Wiese and Binning, 1987). A comparison of the percentage shoot emergence during the period and the estimated T_b appears to show good agreement in this regard (Table 2.2). In this analysis we also introduced the parameter DD_{emerge}. Once the T_b is established the similarity of the DD_{emerge} values at each temperature can be used as a further test of linearity, and provides a convenient parameter for quantifying genotypic differences in thermal responses (see Chapter 5, section 5.1.).

The T_b values of 6 °C to 8.6 °C for *Miscanthus* genotypes are at the lower end of the range found for other below ground propagules; Holt and Orcutt, (1996), reported values of 12 °C for *Sorgum halepense,* 11.4°C for *Cyperus rotundus,* and 5.2 °C for *Cyperus esulentus,* while Satorre *et al.* (1996) reported 7 °C for *Cynondon dactylon.* Angus (1981) makes a tentative suggestion that species may be divided into two groups on the basis of T_b; with temperate-adapted species generally having a T_b below 4 °C and tropical-adapted species

generally having a T_b above 7 °C. According to this general distinction *Miscanthus* would be broadly defined as showing tropical-adapted characteristics but positioned in the lower end of the thermal range.

By combining a low T_b with a high thermal response rate for shoot emergence (Table 2.2) Sin-H9 should be the first to develop in spring. Sin-H9 also had the highest LER within the low temperature range tested. Overall this should allow Sin-H9 to begin forming a canopy earlier than other genotypes.

However, Sin-H9 was the least advantageous genotype in terms of increasing leaf extension with temperature; with a Q_{10} for LER of 6.1 compared with 7.2, 8.2, and 11.9 for Sin-H6, Sac-5 and Gig-2 respectively. The pattern seen in Sin-H9 seems to represent a typical cold adapted response with relatively high LER at low temperatures but a lower thermal response, reducing the maximum extension rate when temperatures are optimal (see Chapter 1, section 1.1.1). Given the lower thermal response of LER the other genotypes would be expected to outperform Sin-H9 when temperatures are above about 17 °C. Over the whole season LER and thermal response of LER will be important determinants of canopy area and ultimately of biomass yield (Biscoe and Gallagher, 1977; Potter and Jones 1977; Collins and Jones, 1988; Grace, 1988). Clifton-Brown and Jones (1997) explored the genetic variation in LER and thermal response of LER in 32 *Miscanthus* genotypes. They used a productivity model similar to that described here to explore the effect of varying the thermal response of LER. Using the thermal responses from the 32 *Miscanthus* genotypes they found potential yields ranging from 3 - 23 t ha^{-1} y^{-1} for Irish climatic conditions. The responses were complex and the genotypes with the highest maximum LER were not necessarily the most productive when leaf extension during low temperature periods was considered. The nature of temperature control of leaf extension is considered in Chapters 3 and 4.

Sin-H9 also appears to be the most cold adapted genotype in terms of frost tolerance. Across all genotypes relative growth rates were largely unaffected by exposure to frost periods from -2 °C to -6 °C, but exposure to temperatures below this resulted in a marked reduction in subsequent growth. Differences between genotypes were not large, but Sin-H9 was significantly more tolerant than the other genotypes with an LT_{50} of –9 °C. It was surprising that Sin-H6 was the least tolerant genotype with an LT_{50} of –6 °C given that

this genotype had the highest rhizome frost tolerance (Clifton-Brown and Lewandowski, 2000); evidently shoot and rhizome frost tolerance are unrelated in this case.

The use of controlled freezing and measurement of subsequent survival to estimate LT_{50} has been found to be an accurate, sensitive and reproducible measure of freezing tolerance, and has shown good agreement with survival in the field (Fuller and Eagles, 1978). In this case leaf condition score provided an adequate measure of seedling survival showing a similar pattern to that of growth rate. This indicates damage to the photosynthetic apparatus from ice formation and photodamage during the light-on period (Levitt, 1980). This would appear to offer a convenient method of assessing frost damage in the field.

In terms of frost tolerance all four *Miscanthus* genotypes would be classed as freezing sensitive (Levitt, 1980). An LT_{50} of –8 °C for Gig-2 is similar to that reported by Ziegenhagen, (1995) for *M.* x *giganteus*, and confirms his conclusion that this genotype is sensitive to late frost events. However, the genus can be considered to have some frost resistance with the LT_{50} values lying within the typical range reported for crops grown in temperate climates, -8 °C to –24 °C (Cloutier and Siminovitch, 1982; Stushnoff, 1984), rather than those reported for tropical grasses, –1.8 °C to –4.3 °C (Ivory and Whiteman, 1978). However, such comparisons are not absolute, as growth condition prior to freezing will have a marked effect on LT_{50}.

The measurements of moisture content appear to provide some explanation as to the source of the genotypic variation in frost tolerance; with Sin-H9 having significantly lower values for both moisture content and LT_{50} compared with the other genotypes. Several studies have found an inverse relationship between shoot moisture content and LT_{50} (Levitt, 1980; Stushnoff, 1984; Abe, 1997; Fowler and Limin, 1997; Rapacz, 1998). Indeed, tissue moisture content is considered one of the best characteristics for use in plant screening programmes as it is often a good predictor of field survival (Stushnoff *et al.,* 1984; Fowler and Limin, 1997). The correlation between moisture content and LT_{50} seen here is quantitatively similar to that found in wheat (Abe, 1997).

The accumulation of osmotically active compounds during cold acclimation is often reported to enhance frost tolerance, probably by decreasing the freezing point of water in the tissue (see review by Levitt, 1980).

In this study the absolute values for osmotic potential did not correlate with frost tolerance, however by comparing the values with those measured from plants of similar age in the field (unpublished data) it appears that the degree of adjustment in osmotic potential may correlate with frost tolerance (Table 2.3). This offers support to the view that osmotic adjustment does not function purely by reducing the freezing point of tissue sap, rather the accumulation of particular solutes protects cytoplasmic molecules and membranes from the effects of cell desiccation during freezing (Thomas *et al.*, 1993; McNeil *et al.*,1999; Thomashow, 2001; larcher, 2003).

Of the four genotypes tested Sin-H9 appears the most cold tolerant, with significantly lower T_b, DD_{emerge} and LT_{50} values, compared with the *M.* x *Giganteus* which is the most commonly grown genotype. The productivity model suggests that these differences in growth characteristics could have a large impact on final yield. Under the conditions typical at the German field site Sin-H9 is predicted to emerge about 35 days earlier than Gig-2. Clifton-Brown *et al.,* (2001) did not detect such large genotypic differences in emergence times during monthly measurements of plant height, but they suggest monthly measurement intervals were insufficient for this purpose. The model results show the earlier emergence of Sin-H9 would result in a longer growing season and slightly higher yields on average. However, early emergence on its own results in a greater variation in yield from year to year as damage due to late frosts becomes more prevalent. When the early emergence is combined with the extra tolerance to frost as found in Sin-H9, the average yield is further increased and the yearly variation is much reduced. Perhaps more important than the increase in average yield is the improvement in tolerance to infrequent severe weather events. In harsher weather conditions the lower T_b, DD_{emerge} and LT_{50} can produce considerably higher yields e.g. in 1991 when severe frosts occurred in late April the Sin-H9 traits resulted in a 26% higher yield. This shows that incorporating theses cold adapted traits will become increasingly important in establishing *Miscanthus* genotypes in colder and more variable climates.

It should be stressed that the model used here retains the advantageous traits from *M.* x *giganteus* such as the high shoot density, RUE and rapid leaf extension at optimal temperatures. The improved yields seen here could only be achieved by combining the 'best' traits from the two genotypes. Clifton-Brown *et al.*, (2001) highlight the need for future breeding programmes to select for *Miscanthus* genotypes better suited to cooler climates. In particular pointing to growth characteristics that will increase the quantity of radiation intercepted during the growing season. Consequently, a primary selection criteria would be for high LER at the beginning of the growing season; including low sensitivity of LER to suboptimal temperatures (Clifton-Brown and Jones, 1997; Jorgensen and Muhs, 2001). The results seen here suggest that additional yield gains could be achieved by breeding for emergence at low temperature as has been successful achieved for germination of maize, beans, tomatoes and cotton (Seefeldt *et. al.*, 2002). Selection for frost tolerance has not always been successful in cereal crops such as wheat and barley, in part due to the lack of genetic variability present in such crop species (Stushnoff, 1984). This problem is not expected in *Miscanthus* as a large number of ecotypes have been recorded in native habitats over a wide range of climates, and many genotypes have already been collected (Numata, 1969; Numata, 1974; Earnshaw *et al.*, 1990; Weng and Ueng, 1997; Deuter and Abraham, 1998).

The general tolerance of Sin-H9 to colder temperatures appears to be reflected in the results of the field trials from across Europe, Table 2.4 shows the yield two years after planting in five European sites, following a latitudinal and temperature gradient. It is not possible to draw comparisons across all sites as *M.* x *giganteus* genotypes did not survive the harsher climate in Sweden and Denmark. Nonetheless there appears to be a general trend with *M.* x *giganteus* performing better in the warmer sites while Sin-H9 and the other *M. sinensis* hybrids are less temperature sensitive performing relatively better in the cooler climates. In Germany the advantageous traits identified in Sin-H9 do not appear to translate into high productivity. Though the two genotypes produced similar yields for the first two years in the third year the yield of Sin-H9 fell below that of Gig-2 and of the other *M. sinensis* hybrids Clifton-Brown *et al.*, 2001). It has been suggested that this lower yield is due to late senescence in autumn, which may reduce translocation from the shoots, and therefore reduces the reserves in the rhizome weakening the growth in the following year (Clifton-Brown *et al.*, 2001). This highlights the need for careful

screening and selection for a range of traits, which are beneficial at each stages of the growing season.

The data from the European field trials (Table 2.4) shows that several *M. sinensis* hybrids have the ability to yield well in the cooler northern sites. Given the poor yields of Sin-H9 in later years and the low shoot frost tolerance seen in Sin-H6, perhaps screening similar to that employed here should be used to investigate a wider range of *M. sinensis* hybrids. Sin-H10 in particular appears to show a tolerance to low temperatures but does not exhibit the late senescence seen in Sin-H9 (Clifton-Brown *et al.*, 2001).

The information from this study combined with the assessments of *Miscanthus* winter frost tolerance (Clifton-Brown and Lewandowski, 2000), and LER (Clifton-Brown and Jones, 1997), provides crucial information for future breeding programmes. Identifying the low leaf frost tolerance of Sin-H6 is important, as this genotype had shown promise exhibiting the highest rhizome frost tolerance (Clifton-Brown and Lewandowski, 2000). Sin-H9, which had the highest leaf frost tolerance here, also exhibited higher rhizome frost tolerance than Gig-2 and Sac-5 (Clifton-Brown and Lewandowski, 2000).

Overall, within the range of genotypes currently available *M.* x *giganteus* remains the most productive in much of northern Europe, but its vulnerability to late frosts may limit its range in colder climates, *M. sinensis* hybrids would appear to be promising candidates for developing a *Miscanthus* genotype suited to such climates.

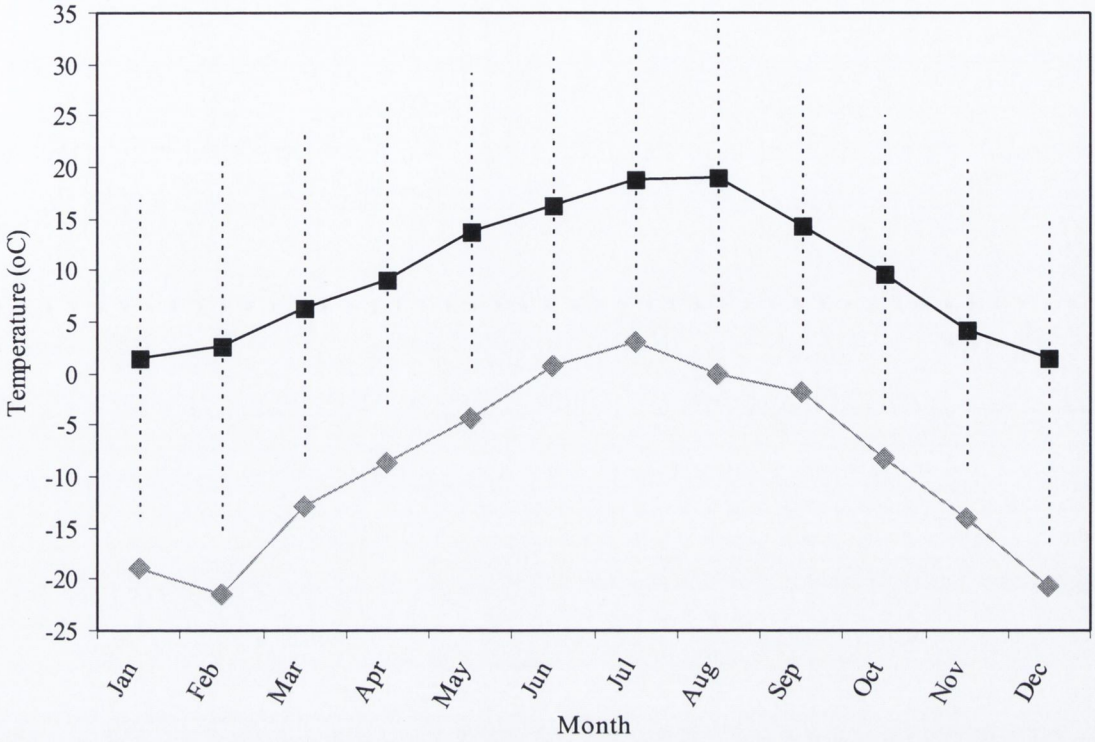


Figure 2.1 Mean monthly temperature and temperature range at the German field site from 1988-1998. Black squares show temperatures measured 2 m above ground, grey diamonds show minimum temperatures measured at the soil surface.

Table 2.1 Details of the four selected *Miscanthus* genotypes.

Name	Identification*	Ploidy	Acquisition code	Additional information
M. x giganteus	Gig-2	3n	ILP53	Hybrid of *M. sacchariflorus* and *M. sinensis* (Hodkinson *et al.*, 1985), No. 16.21 (Greef *et al.*, 1997)
M. sacchariflorus	Sac-5	4n	MATEREC11	Indigenous to sub-tropical areas in Asia, (Matumura *et al.*, 1985)
M. sinensis hybrid	Sin-H6	3n	GOFAL7	Hybrid of two *M. Sinensis* populations from cool temperate Asia, (Deuter, 1998)
M. sinensis hybrid	Sin-H9	2n	JESEL78	Hybrid of two *M. Sinensis* populations from cool temperate Asia, (Deuter, 1998). Considered to be a '*stay-green*' genotype (Clifton-Brown *et al.*, 2001)

* Identification as used by the European Miscanthus Improvement project (Lewandowski, 1998).

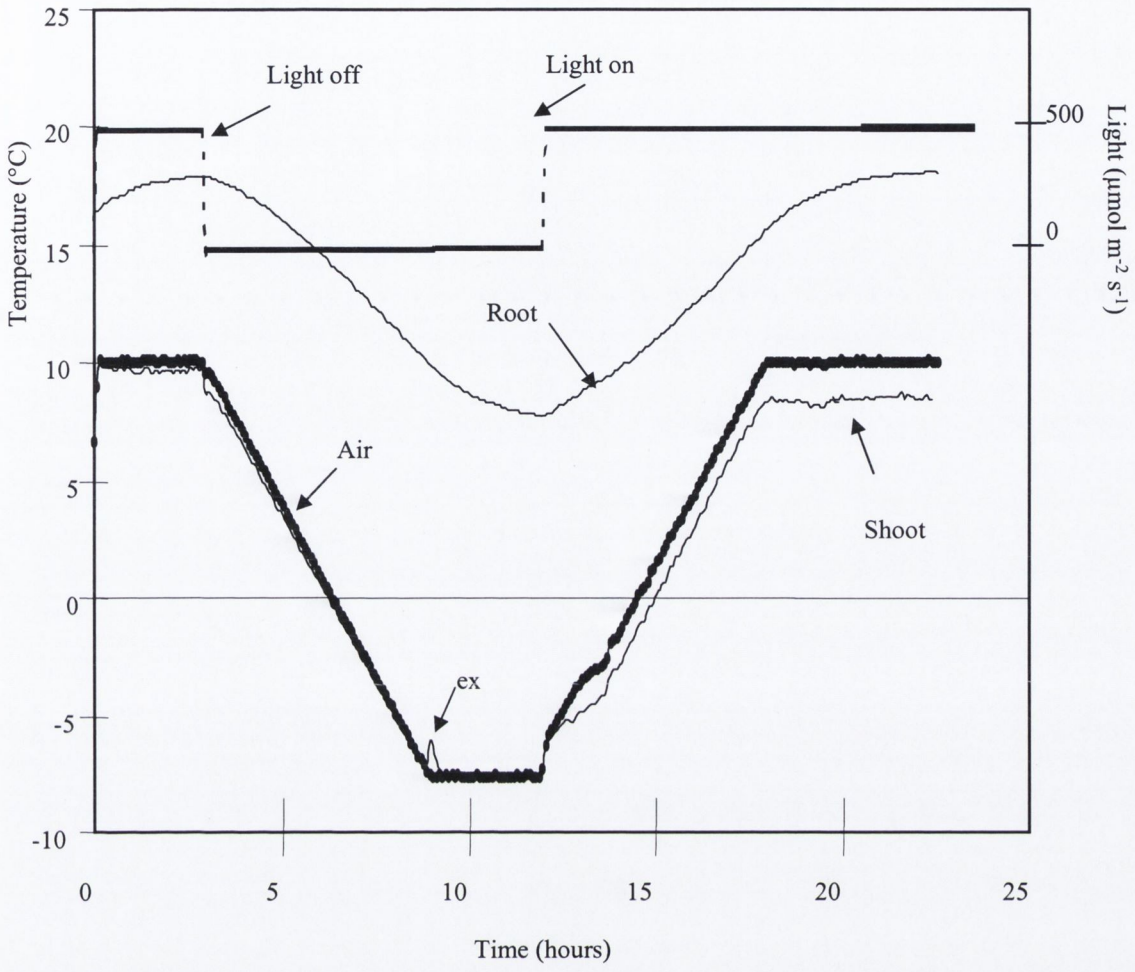


Figure 2.2 Output from thermocouples and PAR sensor during a cooling cycle for the leaf frost treatments: indicating rate of cooling, shoot temperature, the temperature of the roots within insulation, the exotherm produced during ice crystallisation (ex), and the timing of light and dark periods.

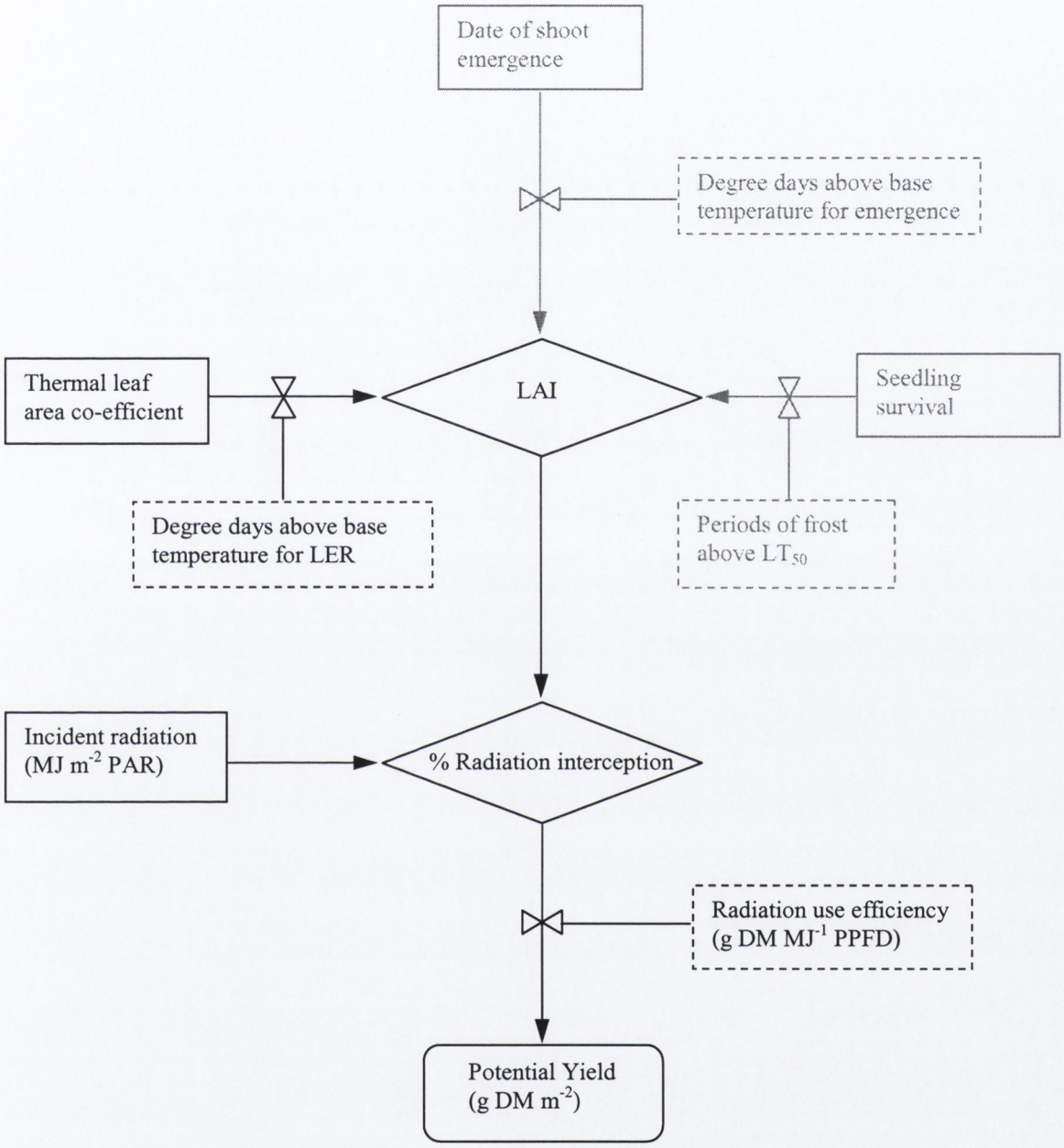


Figure 2.3 The *Miscanthus* productivity model: showing linkages and modules. Additions to the model introduced here are shown in grey. For a given stand leaf area index (LAI) is determined by three factors; (1) date of shoot emergence as defined by the base temperature and degree day requirement for emergence; (2) the thermal leaf area co-efficient as defined by the thermal response of leaf area expansion and shoot density; and (3) seedling survival as defined by the LT_{50} of spring shoots. The LAI in turn determines the percentage of the incident radiation that is intercepted, and the radiation use efficiency determines the amount of this intercepted radiation that is converted into biomass yield. In a given year the impact of each factors is modulated by daily temperature and radiation values. (After, Clifton-Brown *et al.*, 2001).

Table 2.2 The effect of temperature on shoot emergence parameters of four *Miscanthus* genotypes. Different letters indicate significant differences calculated from LSD post-hoc test (P>0.05).

	% Shoot Emergence					Thermal Response Parameters				
	7 °C	9 °C	11 °C	13 °C	15 °C	T_b	slope	r^2	DD_{emerge}	
Sac-5	0	10	50	80	90	8.6	0.008	0.92	118 ± 11	ab
Gig-2	0	40	70	80	90	8.5	0.010	0.99	114 ± 1	b
Sin-H6	10	50	100	100	100	7.6	0.011	0.90	90 ± 11	bc
Sin-H9	60	70	80	70	90	6.0	0.014	0.96	69 ± 7	c

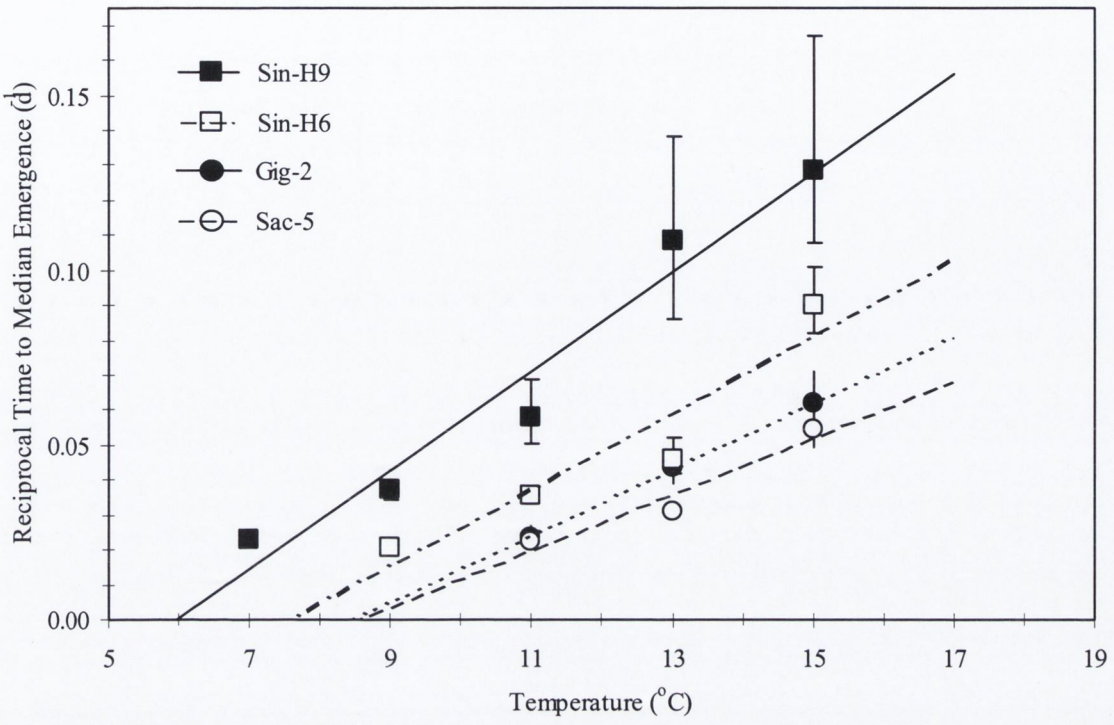


Figure 2.4 The effect of temperature on reciprocal time to median shoot emergence (emergence rate) for four *Miscanthus* genotypes, bars are 95% C.I. of Probits (n=10). The x intercept gives the base temperature for emergence as presented in Table 2.2.

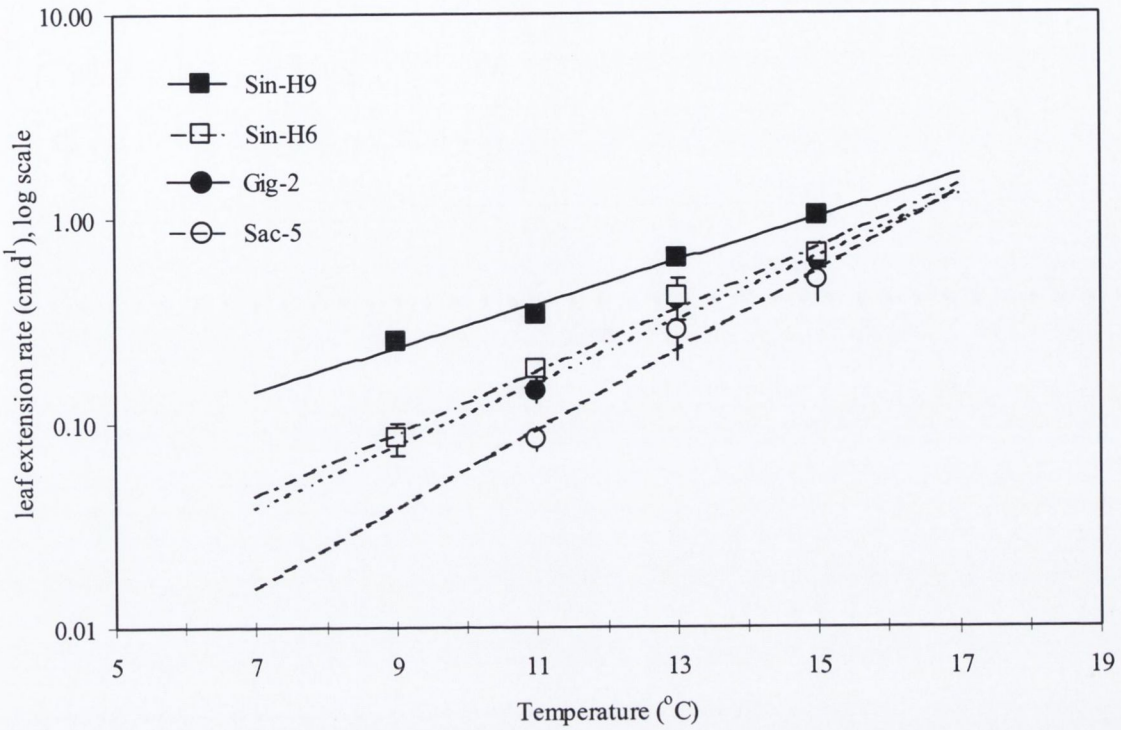


Figure 2.5 The effect of temperature on leaf extension rate for four *Miscanthus* genotypes, bars are s.e. (n=10). Best fit regression models were: Sin-H9 $y=0.0262\ e^{0.2434x}$, Sin-H6 $y=0.0039\ e^{0.3485x}$, Gig-2 $y=0.0032\ e^{0.354x}$, Sac-5 $y=0.0007\ e^{0.4458x}$.

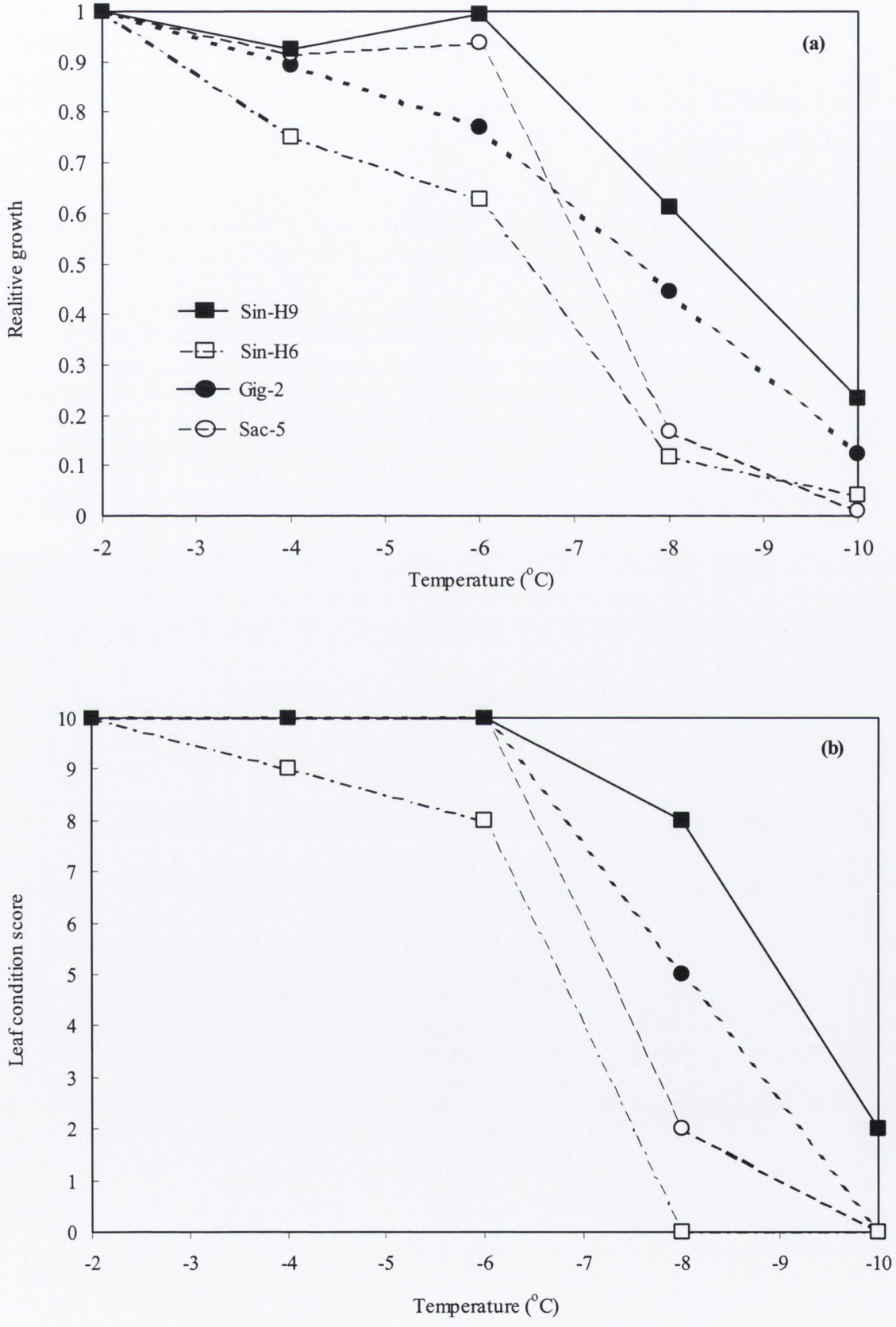


Figure 2.6 The response of four *Miscanthus* genotypes to exposure to a range of below freezing temperatures: (a) Growth rate of leaves measured one week after exposure (rates are expressed relative to plants treated with -2 °C), and (b) Leaf condition score measured one week after exposure.

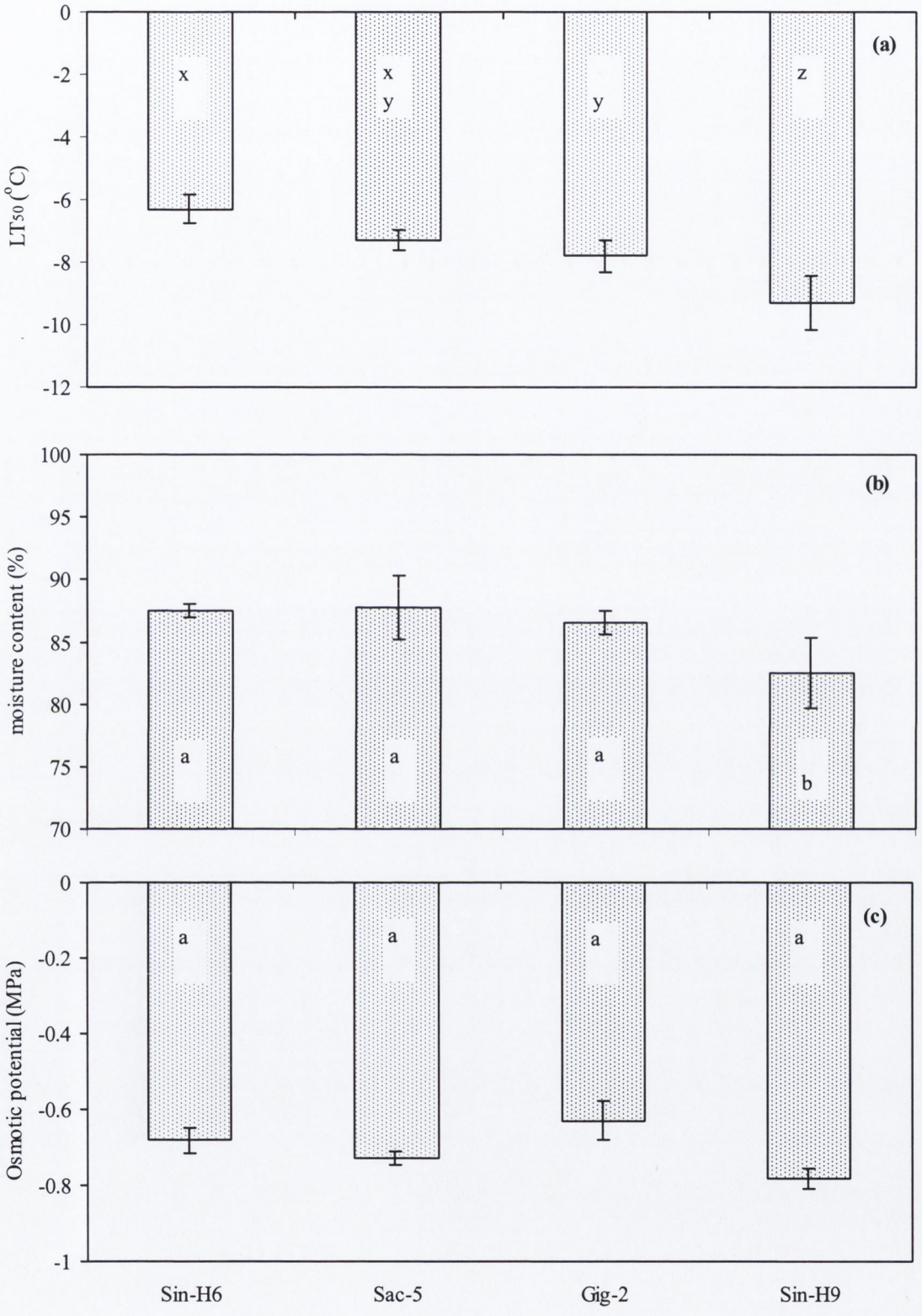


Figure 2.7 Genotypic variation in characteristics related to frost tolerance in prehardened shoots of four *Miscanthus* Genotypes: (a) LT_{50} (lethal temperature at which 50% of shoots are killed); (b) Leaf moisture content; (c) Sap osmotic potential. Shoots were prehardened at 12 °C for 8 d. Different letters indicate significant differences at $P < 0.05$, calculated by the 95% C.I. of the probit analysis (a), or the Scheffe post-hoc test (b) and (c). Bars are 95% C.I. (a), or s.e. (b) and (c) (n = 6).

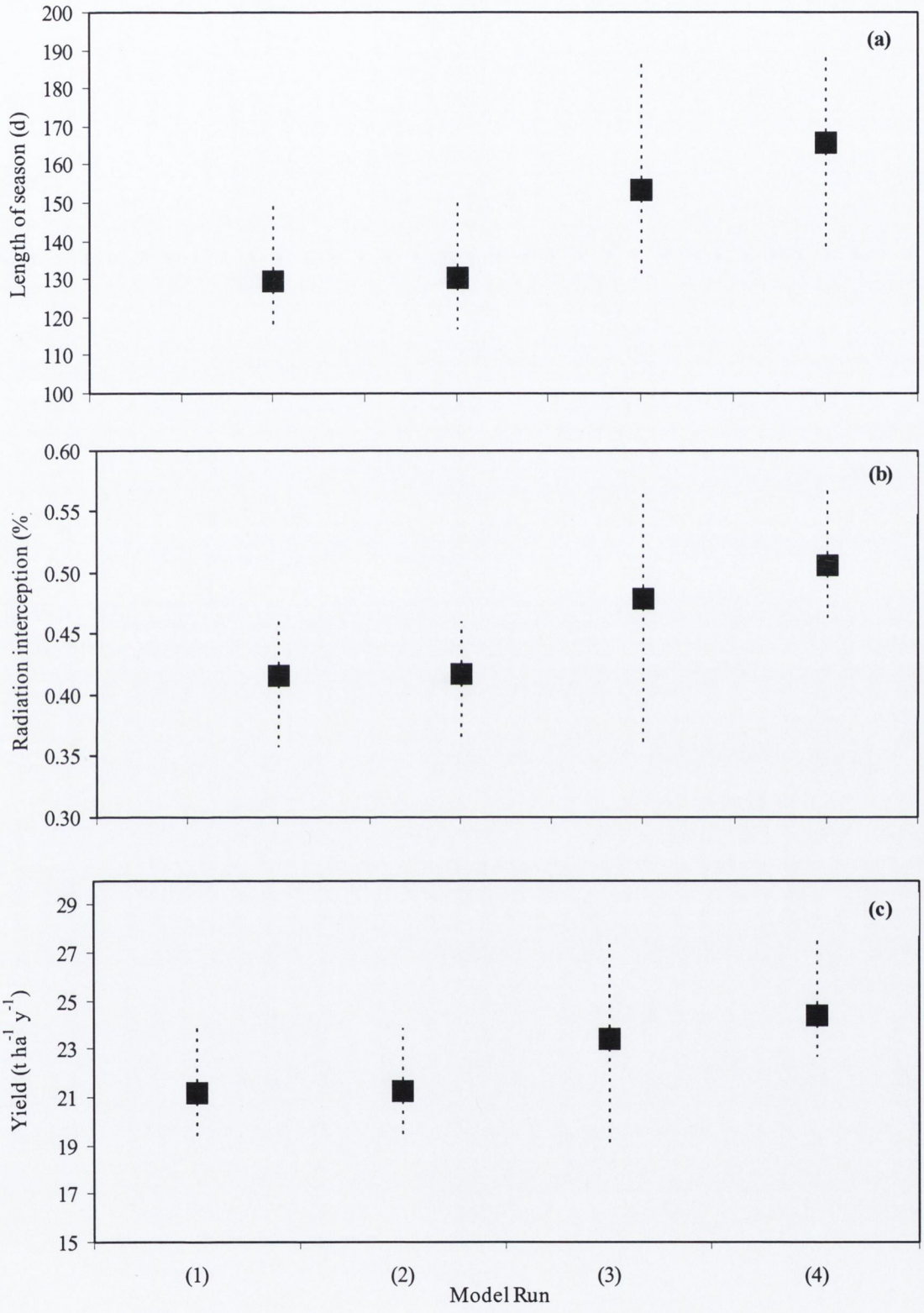


Figure 2.8 Model outputs for: (a) length of growing season; (b) percentage of available radiation intercepted; (c) predicted yield. Each run uses a different set of parameters for frost tolerance (LT_{50}) and emergence (T_b and DD_{emerge}). Parameters are taken from Table 2.2 and Figure 2.6: (1) as for Gig-2; (2) as for Gig-2 with LT_{50} of Sin-H9; (3) as for Gig-2 with T_b and DD_{emerge} of Sin-H9; (4) as for Sin-H9. Data are result of model using 10 y of daily weather data from a site in Germany, with a harvest date of August 20th (see text for details). Bars are range over the 10 y.

Table 2.3 Osmotic potential (MPa) of four *Miscanthus* genotypes cultivated in the 'Field' (i.e. at the Germany site; measured April 1999, Clifton-Brown, unpublished data), compared with those 'Acclimated' (i.e. data as in Figure 2.7; following 8 d at 12 °C), and 'Difference' (i.e. the difference between Field and Acclimated data). Genotypes are arranged in order of increasing frost tolerance (LT_{50}) as shown in Figure 2.7.

	Field	Acclimated	Difference
Sin-H6	-0.650	-0.631 ± 0.051	0.019
Sac-5	-0.550	-0.727 ± 0.018	-0.177
Gig-2	-0.425	-0.681 ± 0.033	-0.256
Sin-H9	-0.450	-0.783 ± 0.026	-0.333

Table 2.4 Autumn yield (dry matter t ha^{-1}) of *M. x giganteus*, *M. sacchariflorus*, and five *M. sinensis* hybrids at five sites in Europe. Yield data is from 1999 two years after planting the final year for which biomass data was collected (Clifton-Brown *et al.*, 2001).

	Sweden	Denmark	England	Germany	Portugal
Temperature (°C)*	13.8	13	14.4	14.9	20.2
Sac-5	0.0	1.4	11.1	12.6	35.2
Gig-2	0.0	0.0	16.8	24.3	36.4
SinH-6	19.3	16.4	15.7	20.0	27.2
SinH-7	11.0	10.4	17.7	17.0	40.9
SinH-8	24.7	15.9	6.5	19.2	21.0
SinH-9	14.2	0.9	15.8	10.3	26.3
SinH-10	20.5	18.2	14.0	19.1	20.3

* Mean air temperature from April to September

Chapter 3: Biophysical control of leaf extension at low temperatures

3.1 Introduction

In temperate climates where water and nutrient availability are generally adequate, low temperature will often be the factor most limiting for plant growth (Gallagher and Biscoe, 1979c, Ong and Baker, 1985; Grace 1988; Pollock and Eagles, 1988; Gastal *et al.*, 1992). Identifying the nature of this limitation remains a key question for those seeking to increase plant productivity in cold wet climates (Stushnoff *et al.*, 1984; Pollock *et al.*, 1993).

Plant productivity is determined by the amount of photosynthesis carried out by the canopy during the growing season and this in turn is dependent on the rate of photosynthesis, and on the rate at which leaves grow to intercept radiation. Physiological comparisons have indicated that leaf extension is considerably more temperature sensitive than the photosynthetic processes (Grace, 1988; Korner and Larcher, 1988; Pollock and Eagles *et al.*, 1993). This has been shown in C_3 (Pollock *et al.*, 1993) and C_4 species (Miedema, 1989) and indicates that dry matter production at suboptimal temperatures is likely to be more restricted by the formation of canopy area than by the rate of carbon fixation. As such the response of leaf extension and canopy development to short periods of low temperature may be crucial in determining productivity.

In grasses and cereals the growth of the canopy takes place in the meristem and leaf extension zone (LEZ) at the base of the leaf. Cooling of this basal region results in a reduced leaf extension rate (LER), which can be detected within 20 s (Stoddart *et al.*, 1986). A response over this time scale cannot be due to a limitation in the supply of unexpanded cells from the meristem. Such a rapid responses also makes it unlikely that long distance signalling is involved, at least in the short-term. Thus cooling appears to affect the process of cell expansion directly. Lockhart (1965) provided an empirical model that can be used to analyse the cell expansion process:

$$RER = \phi(\Psi_p - Y)$$

Where ϕ, the wall extensibility ($mm.mm^{-2}\ MPa^{-1}\ s^{-1}$) determines the rate of irreversible flow of the wall for a given stress, Ψ_p is the turgor pressure (MPa) which provides the

stress, and Y is the yield threshold of the wall below which no extension will occur (MPa). In this model turgor pressure provides the driving force for wall yielding, while wall yielding provides the driving force for water uptake (Cosgrove, 1986). Control of cell expansion rate can therefore occur through changes in turgor pressure or wall properties.

LER has been observed to change in line with turgor pressure when plant water status is altered; such as when water supply is limited, during drought (Matthews *et al.*, 1984), salt stress (Cramer and Bowman, 1991), or root cooling (Pardossi *et al.*, 1994); or when transpiration load is altered by manipulation of vapour pressure deficit (Taylor and Davies, 1986) or by leaf shading (Shackel *et al.*, 1987). Short-term fluctuations in LER often result from declines in turgor pressure, with both turgor pressure and LER being restored, at least partially, through stomatal closure (Pardossi *et al.*, 1994) and/or osmotic adjustment (Cramer and Bowman, 1991).

Altered wall properties have also been found to correlate with differences in LER brought about through a range of growth conditions such as water stress (Bunce, 1977; Matthews *et al.*, 1984; Wu *et al.*, 1996; Thomas *et al.*, 1999), transition from dark to light (Cosgrove, 1989; Cosgrove and Li, 1993; Parvez *et al.*, 1996), variation in gravity (Soga *et al.*, 1999; Hoson *et al.*, 2002) and application of hormones such as Auxins, Gibberellins, Brassinosteroid, ABA and Fusicoccin (Cleland, 1971; Cosgrove, *et al.*, 1987b; Kutschera and Schopfer, 1986; Cosgrove and Sovonick-Dunford, 1989; Zurek *et al.*, 1994). Theses changes in wall properties were detected using a range of techniques. A distinction must be made between those techniques that measure the extension properties of killed tissues e.g. Instron analysis, and those carried out *in vivo* or in metabolically active tissue, e.g. biochemical creep analysis (see Chapter 1, section 1.2.4 and Cosgrove, 1993). This distinction is particularly important for short-term effects when the metabolic component of cell expansion may be more significant.

Chilling of the roots has been shown to reduce turgor pressure in expanding cells and so inhibit LER in the short-term (Pardossi *et al.*, 1994) and in the long-term under severe chilling (Watts, 1972a). This indicates that cell expansion is limited by water influx at suboptimal temperatures. However, root chilling does not reduce turgor pressure in the

root extension zone where expansion is also inhibited indicating that temperature reduces the extensibility of the cell wall (Pritchard *et al.*, 1990a).

Woodward and Friend (1988) studied the response to long-term chilling in *Poa* plants from three different altitudes (i.e. adapted to different thermal regimes). They found no drop in turgor pressure when the whole plant was cooled. Instead they showed reduced LER in the cold grown plants correlated with changes in wall plasticity as measured by Instron analysis, with the largest fall in plasticity seen in the most temperature sensitive lowland species. However, the changes in 'Instron plasticity' do not completely explain the reduced LER at low temperature as in the high altitude species LER falls by approximately 40 % between 18 °C and 5 °C with no change detected in Instron plasticity.

Thomas *et al.* (1989) investigated the effects of short-term cooling on *Lolium temulentum* L. seedlings and found no change in turgor when LEZ temperature was varied from 20 °C to 2 °C. They conclude that wall properties are key in the control of extension rate following cooling although in subsequent studies Thomas (1990) failed to detect any difference in Instron plasticity following 30 minutes treatment at 5 °C, even when measurements were carried out at 5 °C.

It appears that the short-term effects of cooling are not explained by changes in Instron plasticity or turgor pressure. Yet these immediate reversible effects may have a dominant role in determining overall leaf growth in the field. For example, in the mid-altitude species examined by Woodward and Friend (1988) Instron plasticity appears to account for the reduced LER when plants are grown at low temperature, however, LER is even more restricted by short periods of low temperature in the field (Woodward *et al.*, 1986). This is despite the fact that the results of Thomas (1990) suggest no change in Instron plasticity over this timescale.

In this Chapter the short-term effects of cooling on turgor pressure and wall properties are investigated in a C_4 grass that shows high temperature sensitivity in LER. Wall properties are measured using long-term creep analysis on freeze/thawed tissue to allow assessment of thermal response in metabolically active tissue. It is hypothesised that the reduced LER during short-term cooling will correlate with a fall in turgor pressure and/or wall extensibility.

3.2 Materials and Method

3.2.1 Plant material

Initial trials were carried out using *Miscanthus* seedlings, however this material was found to be unsuitable for use with the pressure probe. Two problems were identified: Firstly the LEZ was difficult to access through the ridged tightly bound outer leaves; Secondly the expanding cells tended to block the microcapillary, this may have been due to a wax on the surface of the epidermis or due to characteristics of the wall tissue itself.

A decision was made to use maize, a commonly studied C_4 grass, for the remainder of biophysical experiments. A chilling sensitive variety of maize (*Zea mays v. Penjalina*) was used for all subsequent experiments. For the extensiometer experiments maize seedlings were grown in vermiculite and watered with ½ strength Hoagland's solution (Hoagland and Arnon, 1951), while for the pressure probe measurements seedlings were germinated on filter paper and grown in aerated ½ strength Hoagland's solution. In each case the seedlings were kept in a growth cabinet (Fitotron, Sanyo) at 21 °C, with relative humidity at 65%, and a 14 h light period at 300 μmol m^2 s^{-1} Photosynthetic Photon Flux Density (PPFD). Measurements were carried out on the second leaf after 14 days when LER at 21 °C was 0.962 ± 0.042 mm h^{-1} (s.e. n=22).

3.2.2 Determining the position of the leaf extension zone

Monocots grow from the base the leaf, with the expanding cells covered by older leaves and the outer sheath. In order to determine the position of the LEZ a pin prick experiment was carried out as described by Kemp (1980). Fine needles were used to pierce holes horizontally through the tissue starting at the seed, the needles were positioned in a ridged frame at 2 mm intervals. The plants were returned to the growth cabinet and after 12 h the position of the holes was again measured. The outer leaf was removed and the position of holes on leaf 2 recorded. The distance between the holes was measured using a ruler under a stereo microscope. A plot of expansion between the holes against the distance from the seed showed the zone of maximum cell expansion, i.e. the LEZ.

3.2.3 Leaf extension measurements

LERs were measured using auxonometers made from Linear Variable Displacement Transformers (LVDTs) with a stroke length of 100 mm (Type DC/50; Schlumberger, Bognor Regis, UK). These were mounted on weighted retort stands within the growth cabinets. The LVDTs were attached by a clamp to leaf 2, via a Kevlar thread (Type 989, 440 Dtex; Dupoint, Derry, N. Ireland) that ran over a Teflon pulley. The thread was weighted on the plant side to balance the weight from the LVDT core, an additional 1.5 g was then added to the core to overcome friction. LVDT output was averaged every 30 s and recorded with a datalogger (Type 21X, Campbell Scientific, Leicestershire, UK). LER was calculated from the slope to leaf length by time using a linear model ($r^2 > 0.95$).

3.2.4 Pressure Probe technique

Single plants were transferred from the cabinet to a 100 ml plastic container holding ½ strength Hoagland's solution. The seed was held in the container by 1 cm disk of polystyrene, which served to isolate the root temperature from that of the shoot. The nutrient solution was aerated throughout the experiment except during the turgor pressure measurement.

In order to access the expanding cells in the LEZ it was necessary to cut away a section of the outer sheath and cotyledon leaf. This tissue was removed in strips using a fresh razor blade. A cut was made horizontally starting above the LEZ at the point where the outer leaf overlaps with itself. The tissue was removed by pulling downwards and away from the inner leaves. This allowed the removal of the outer tissue without touching the inner leaf with the blade. A section of approximately 3 x 8mm of outer tissue was removed. This modification of the window cutting method used by Thomas (1990) was found to be less disruptive for these seedlings with LER following cutting, on average 51 ± 12 % of controls (s.d. n=8). The exposed section of the LEZ was kept moist throughout the experiment with droplets of distilled water. Transpiration load was minimised by covering the leaves with a hood consisting of a layer of wet tissue paper in a black polytene bag.

A 25 mm section at the base of each plant was held in a collar constructed from aluminium foil, which could be adapted to fit the dimensions of each plant. A 10 x 3 mm

section on one side of the collar was removed to allow access to the LEZ. The aluminium collar was connected to a cold plate (40 x 50 mm), which was part of a commercial stage Peltier cooler (Pelcool, Thermoelectric division, C. W. Brown Ltd, UK). The hot side of the Peltier plate was cooled by a water block connected to a circulating water bath (Haake F3/C, Berlin, Germany). Thermocouples (Type T, RS components, Dublin, Ireland) were placed inside a collar adjacent to the seedling and in the plastic container alongside the roots. Preliminary tests confirmed that the LEZ temperature could be held constant over long periods without affecting the root temperature.

The turgor pressure of individual epidermal cells was measured directly using a pressure probe as described by Tomos (1999) (see Figure 3.1). The pressure probe consisted of an oil-filled microcapillary sealed into a Perspex chamber that contained a motor driven piston and a small solid-state pressure transducer (PDCR 200; Drunk Ltd, UK). The microcapillaries were produced from glass tubes (1 mm, GC100-15; Clark Electromedical, UK), which were pulled to a tip diameter of less than 1 μm using a conventional electronic solenoid-based puller (CFP Microelectrode puller, 230 V; Searle Bioscience, UK). The microcapillary was opened by abrading the tip on a micro-forge, so that the final tip diameter was 1-3 μm. The pressure probe was filled with low viscosity silicone oil (AS4; Wacker-Chemie, Germany), which was filtered and degassed prior to use.

The pressure probe was mounted on a micromanipulator (Ernst Leitz Wetzler; Leitz, UK) and measurements made with the aid of a stereomicroscope (M8; Wild Heerbrugg Ltd, Leitz, UK). Illumination was supplied via a fibre optic system (Intralux 5000; Volpi AG, Leitz, UK).

When the microcapillary was inserted into a cell, turgor pressure forced cell sap outward into the capillary, forming a meniscus between the sap and the oil. The force provided by the sap caused compression of some pressure probe parts (largely the oil and rubber seals), such that no initial change in pressure was detected. The motorised piston inside the Perspex chamber was then used to increase the pressure of the oil and push the meniscus back towards the cell surface. The sap was held at the cell surface by increasing and decreasing the pressure provided by the piston. When the meniscus was stationary the pressure of the oil inside the chamber was equal to the pressure in the cell. This

pressure was recorded as the cell turgor pressure. The pressure was monitored over several minutes to ensure that the cell was not damaged and the meniscus was moved back and forth to ensure that the capillary was not blocked.

The pressure sensor was calibrated as mV/MPa against a column of mercury in a firm nylon tube lifted to various heights (76 cm = 0.1 MPa).

3.2.5 Creep measurements

For creep measurements, a segment of the LEZ tissue was held under constant load in an extensiometer and the extension measured over several hours. Samples were prepared using a protocol similar to that used for cucumber hypocotyls, as described in Cosgrove (1989) and subsequent studies (McQueen Mason and Cosgrove, 1992; Whitney *et al.*, 2000).

The temperature controlled extensiometer consisted of a brass chamber which contained a buffer solution and supported a set of clamps which held the LEZ segment (Figure 3.2). The upper clamp had a fixed position while the lower clamp was freely movable and incorporated an LVDT core to measure extension of the segment. A 20 g weight was attached below the LVDT to provide force for the extension of the wall tissue. Temperature control was achieved by cooling the solution in the chamber with a thermoelectric cooling plate attached to the brass chamber with thermal paste. The temperature of the buffer was monitored with a fine wire thermocouple within the chamber adjacent to the LEZ segment.

The LEZ segments were prepared in a way which removed turgor pressure and excess fluid from the tissue while maintaining protein activity in the cell walls. Fourteen days after planting, seedlings were removed from pots and placed immediately in a freezer at -20 °C, where they were stored for no more than 5 days. After transferring seedlings from the freezer the outer sheath and cotylodenous leaf were removed and a 10 mm segment of LEZ was taken for analysis. The segment was abraded by washing the surface with a thick slurry of 200-450 mesh silicon carbide (Carborundum) which allowed the buffer solution to freely access inner tissue. Segments were then rinsed with distilled water and placed on blotting paper between two glass slides under a 40 g weight for 5 minutes, to remove excess fluid. The segment was then placed in a template specially constructed to

allow clamping of the tissue with 5 mm between clamps, and to ensure the two clamps were kept parallel. For control measurements, proteins in the cell wall were inactivated by boiling the segments in water for 10 minutes prior to being placed between the glass slides.

The clamp holding the LEZ tissue was transferred to the extensiometer, taking care not to apply excess force to the tissue during installation. Once in the extensiometer a weight was added to the lower clamp resulting in a total 5 g force that served to remove slack from the system. The circumference of the lower clamp was sealed with vacuum grease to allow the chamber to be filled with a buffer solution. The buffer was 1.3 ml of 50 mM potassium acetate, pH 4.5, and was applied through a hole in the front of the chamber using a syringe.

After 10 minutes the weight was increased to 20 g and extension monitored for 7 h. After 1.5 h temperature treatments were applied using a sequence of 14 °C, 7 °C and 21 °C. The steady state temperature was reached within approximately 10 minutes and each temperature was held for 30 minutes.

The output of the LVDT and thermocouple was averaged every 30 s and recorded with a datalogger (21x, Campbell Scientific). For initial analysis the rate of extension was taken as the slope of a linear regression for the last 20 minutes at the steady state temperature.

3.3 Results

3.3.1 Response to cooling

The LEZ was found to extend for approximately 20 mm from the base of the leaf. Cells appear to expand at an accelerating rate over the first 8 mm of the LEZ, after which there was a slow decline in the expansion rate (see Figure 3.3). This pattern has also been observed in the LEZ of tall fescue (Schnyder *et al.*, 1988), wheat (Thomas, 1990), ryegrass (Thomas, 1990), barley (Fricke, 2002), and in the root extension zones of several cereals (Spollen, *et al.*, 1993; Pritchard , 1994). Subsequently a 5 mm section from 6 to 11 mm from the seed was taken as the zone of maximum extension and was targeted for the pressure probe and creep measurements.

Cooling of the LEZ alone caused a marked drop in leaf extension (Figure 3.4). LER was seen to respond in less than 30 s (the measurement interval used), with a steady LER being established as soon as the new temperature set point was reached. The thermal response of LER over the three temperatures resulted in Q_{10} values of 5.1 between 7 °C and 14 °C, and 4.3 between 14 °C and 21 °C (Figure 3.5). An exponential model provides an adequate fit over the temperature range used. However, the actual base temperature seen when seedlings were cooled over a wider temperature range appears to be higher than that predicted from the three temperature points.

3.3.2 Turgor pressure

At 21 °C the turgor pressure of the expanding cells was 0.32 ± 0.05 MPa, significantly lower than that of the mature non-expanding cells at 0.42 ± 0.08 MPa (Figure 3.6).

Cooling the LEZ had no significant effect on turgor pressure in the expanding cells, with an overall mean of 0.32 ± 0.04 MPa (Figure 3.7). The standard deviation at 0.04 MPa reflects variation in the turgor pressure from cell to cell as well as error during measurement, as such it represent the resolution of the turgor pressure measurement presented.

3.3.3 Creep analysis

Creep analysis of the LEZ segments produced a viscoelastic response which dominated for the first hour and continued to form a significant component up to 2 h after application of the force (Figure 3.8).

The remainder of the curve is dominated by an approximately linear extension persisting for at least 6 h, here called 'biochemical creep' to distinguish it from the viscoelastic response analysed in traditional rheological assays (see Chapter 1, section 1.2.4). Although the biochemical creep was seen to persist over prolonged periods, in many cases the rate continued to decline beyond 2 h (Figure 3.8 inset.). It is unclear whether this represents a continual contribution of the decaying viscoelastic response or whether the rate of biochemical creep is itself falling over time (e.g. due to the disrupted

metabolism). For this reason the temperature treatments were applied using the lower temperatures first to avoid any false positives in the Q_{10} due to greater extension rates in the earlier parts of the run.

The biochemical creep was seen to respond immediately to temperature change (Figure 3.9). In many cases the segments appeared to shrink during cooling and expand rapidly during heating, probably due to expansion and contraction of the apparatus itself. Once a new stable temperature was reached the creep rate was seen to change in line with temperature. In the heat inactivated segments the extension was considerably lower particularly towards the end of the treatment cycle.

In the active tissue there was a clear positive correlation between temperature and creep rate (Figure 3.10). In the heat inactivated tissue there appeared to be some response to temperature with the rate at 7 °C slower than that at 14 °C. However the pattern in these segments is dominated by the decline in the creep rate over time resulting in the lowest rates at 21 °C (the last treatment). The heat inactivated tissue had considerably lower extension rates at all temperatures.

A second control run was carried out using active tissue segments but substituting the usual pH buffer for one with 3 M NaCl added to disrupt protein activity. Again the thermal response is greatly reduced with no clear pattern between 7 °C and 21 °C (Figure 3.10).

In order to remove any errors due to a decline in the base creep rate or to effects of temperature change on the apparatus itself, the mean creep values for the heat inactivated tissue were subtracted from those for the active tissue. The extension remaining after this analysis is shown in Figure 3.11, this represents the metabolically mediated element of extension and again shows a clear increase in creep rate as temperature rises, with Q_{10} values of 5.0 between 7 °C and 14 °C and 2.4 between 14 °C and 21 °C.

3.4 Discussion

Cooling the LEZ of maize seedling resulted in an immediate reduction in the LER; as seen in previous studies using a variety of plants. In the past researchers have failed to identify the cause of this inhibition of leaf extension. Here we have shown that the reduced LER is associated with a reduction in the creep rate of freeze thawed tissue from the LEZ. This thermal response was not seen in LEZ tissue that was heat-inactivated prior to testing. The implications of these results are discussed below in relation to control of cell expansion from water entry and from cell wall extensibility.

3.4.1 Water entry, turgor pressure and cell expansion

Low temperature has the potential to slow cell expansion by reducing hydraulic conductance (L) or by increasing osmotic potential (Ψ_s), either of which could restrict water entry to the expanding cells causing a drop in turgor pressure. Cooling of leaf tissue has been shown to reduce hydraulic conductivity in growing (Thomas *et al.*, 1990) and mature cells (Tomos *et al.*, 1981). Temperature effects Ψ_s directly as explained by the van't Hoff relation, and chilling has been shown to cause a small reduction in the turgor pressure (0.03 MPa between 20 °C and 8 °C) of single celled algae (Proseus *et al.*, 2000). Low temperature could also inhibit a plants ability to maintain Ψ_s, again this should be reflected by a drop in turgor pressure (Pritchard, 1994). In this study no change in turgor pressure was seen, indicating that water entry does not limit cell expansion during short-term cooling of this chilling sensitive grass. Similar studies with the more chilling resistant grasses *L. temulentum* (Thomas *et al.*, 1990) and barley (Pollock *et al.*, 1990), and with Pea hypocotyls (Cosgrove and Cleland, 1983), also found no reduction in turgor pressure during short-term chilling.

Likewise, long-term chilling seems to reduce cell expansion without limiting water entry. In fact turgor pressure is higher following chilling, as observed in *Poa* plants after 10 days (Woodward and Friend, 1988) soybean hypocotyls after 2 h (Boyer, 1993), and maize leaves after 48 h (Watts, 1972). Thomas (1990) monitored turgor pressure during chilling of wheat LEZ and found that turgor increased over the first 1 to 2 h.

The maintenance of turgor pressure suggests that hydraulic conductance across the LEZ although reduced is considerably larger than extensibility ($L >> \phi$), as concluded by

Thomas *et al.* (1989) and Boyer (1993). Boyer (1993) suggests that the reduction may be accounted for by the expected increased in viscosity of the cell sap.

Similarly solute supply does not appear to limit water entry during chilling. Boyer (1993) found no change in osmotic potential during cooling over 20 h, while Nakamura *et al.*, (2002) report a clear decrease after 8 h, as does Watts (1972) after 48 h, and Woodward and Friend (1988) after 10 days. Rather than restricting solute supply chilling seems to result in solute accumulation. This may occur because solute supply is less temperature sensitive than other expansion processes, or it may be a mechanism of cold acclimation as seen in mature cells (Levitt 1980; Hughes and Dunn, 1996; Fitter and Hay, 2002).

Watts (1972) and Woodward and Friend (1988) showed explicitly that the decreases in osmotic potential corresponded to an increase in turgor pressure. This provides evidence that cell wall properties are limiting expansion, as only an unyielding wall would result in a turgor increase.

3.4.2 Creep analysis

The creep rate of boiled segments is determined by the mechanical properties of the cell walls and does not appear to be effected by temperature change (Figure 3.10). This is in agreement with previous studies, which have found that the mechanical properties of wall tissue are largely insensitive to temperature treatments applied during the assay, at least within the 5 °C to 25 °C range (Haughton *et al.,* 1967; Cleland, 1971a; Thomas, 1990; Pritchard *et al.*, 1990a).

This contrast with the active tissue, in which creep rate is clearly reduced by cooling. The creep of active tissue is determined by both the mechanical properties of the walls and by biochemically mediated extension in the wall. As seen in Cosgrove (1989) this 'biochemical creep' is inhibited by boiling tissue prior to measurement or by exposure to NaCl during the assay. The biochemical creep rate at 21 °C (Figure 3.10) represents extension of 1.7 % h^{-1}. This compares well with the extension seen in other studies using a similar method, e.g. rice internodes, 0.8 % h^{-1} (Cho and Kende, 1997); oat coleoptiles, 1.8 % h^{-1} (Cosgrove and Li, 1993); and cucumber hypocotyls, 2.4 % h^{-1} (Cosgrove, 1989).

The reduction in the rate of biochemical creep during short-term cooling appears to account for the inhibition seen in LER. The timescale over which biochemical creep responses to cooling is similar to that seen for leaf extension, with creep appearing to respond to temperature instantaneously (Figure 3.9), although it is difficult to analyses activity during the temperature change due to expansion and contraction of the apparatus itself. The thermal response of biochemical creep is similar to that of LER between 7 °C and 14 °C, the rate at 21 °C is lower than would be expected but still shows high temperature sensitivity (Compare Figure 3.5 and Figure 3.11). Thus, temperature appears to have a direct effect on wall properties mediated by the biochemical components of wall loosening.

In this study changes in the extension properties of the wall appear to account for the inhibition of LER during short-term cooling. The failure to detect such changes in wall properties in *L. temulentum* (Thomas, 1990) probably results from the limitations of Instron analysis particularly the use of killed tissue in the assay (see Chapter 1, section 1.2.4 and Cosgrove, 1997). During cooling of maize roots Pritchard *et al.* (1990a) observed similar limitations in the ability of Instron analysis to detect changes in wall properties. Pritchard *et al.* (1990a) found that although long-term chilling of plants resulted in both inhibited cell expansion and reduced Instron plasticity, no change in the plasticity was associated with short-term cooling despite the almost total cessation of expansion. The authors offer the explanation that there are at least two growth-slowing activities, only one of which can be detected by Instron analysis.

Cleland (1984) also commented on limitations with Instron analysis in accounting for short-term effects on growth rate following application of Auxin and KCN. Cleland (1984) proposes that the measurement of plasticity by the Instron method represents those domains in the wall where a wall-loosening event has occurred but has not been eliminated by further biochemical events. In this case, the plasticity measured may reflect the wall properties of the immediate past rather than the current state of the wall as the population of unfixed domains will reflect the sum of past expansion rates. If this is the case the biochemical creep can be taken as a measure of the loosening events that cause the inhibition of LER, while the Instron measurements show the influence of these events over time on one aspect of wall architecture. Thus the effect of chilling on mechanical

wall properties during prolonged cooling (Woodward and Friend, 1988; Tomos and Pritchard *et al.*, 1990a; Nakamura, 2002) may be an indirect consequence of the reduced rate of wall loosening.

However, Cosgrove (1997) argues that the dissimilarities between Instron plasticity and cell expansion rate are too great even for the indirect relationship suggested by Cleland (1984). Cosgrove (1997) concludes that Instron plasticity can only be used as a measure of general differences in wall architecture that may or may not relate to cell expansion. This argument is supported by cases where treatments have resulted in increased Instron plasticity while cell expansion was unchanged or even inhibited (Ruesink, 1969; Coartney and Morre, 1980). This degree of separation between the mechanical properties of the wall and the extension properties of the wall may explain the lack of correlation between growth rate and Instron plasticity discussed by Cleland (1984) and also that seen in during cooling (Thomas, 1990; Pritchard *et al.*, 1990a). In this case, the changes in Instron plasticity during long-term cooling do not necessarily relate to the reduced action of Wall Loosening Factors (WLFs). Instead the reduced Instron plasticity may result from changes in wall architecture that occur as a secondary response to the low temperature stimuli, perhaps related to the development of frost tolerance (see Chapter 5, section 5.4.3).

It is likely that temperature affects cell expansion through two distinct mechanisms: (1) Long-term chilling results in changes in wall architecture which reduce the Instron plasticity (Woodward and Friend, 1988; Pritchard *et al.*, 1990a; Nakamura, 2002); (2) Short-term cooling does not affect wall architecture but inhibits cell expansion through its effect on the process of wall loosening (Figure 3.10).

It is now widely accepted that wall expansion involves the activity of certain WLFs, with expansins being the primary candidate for this activity (Cosgrove, 1999). Expansin activity appears to be essential for the sustained wall creep as seen in this study. The mechanism of expansin activity is not known, but evidence suggests that expansins act in a catalytic way disrupting hydrogen bonds between cross-linking glycans and the cellulose microfibrils they hold together (Cosgrove, 2000a). This process is thought to be driven not by chemical energy but by the mechanical energy provided by turgor pressure. The temperature sensitivity of such a mechanism would come from the activation energy

needed to break hydrogen bonds between the two polymers (McQueen Mason and Cosgrove, 1992; Cosgrove 2000b).

Overall these results suggest that cell expansion at suboptimal temperatures is restricted neither by water supply nor by the mechanical properties of the cell wall. Regardless of whether Instron plasticity measures changes in cell architecture related to past extension or changes that are unrelated to extension, the initial inhibition of cell expansion appears to be mediated through an effect on the activity of WLFs alone. This conclusion diverges somewhat from that of Nakamura (2002) who believes that "temperature modulates the growth rate of plant shoots by affecting mainly the mechanical properties of the cell wall". This view is based on measurements of Instron plasticity following at least 4 h at contrasting temperatures, these results are discussed in Chapter 5, section 5.4.3.

It remains possible that genotypic differences in thermal response can be mediated either through the response of WLFs or through differences in the bonding arrangements of the wall as reflected in the mechanical properties, this aspect is investigated further in Chapter 4.

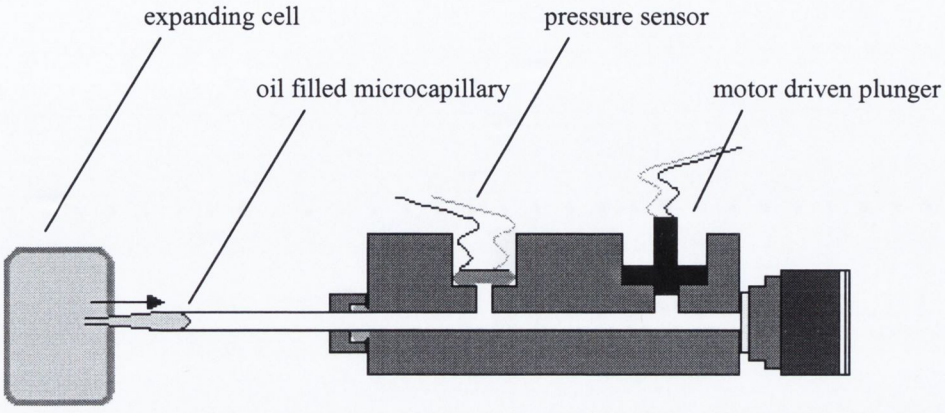


Fig 3.1 Diagram of pressure probe showing principle components. See text for details.

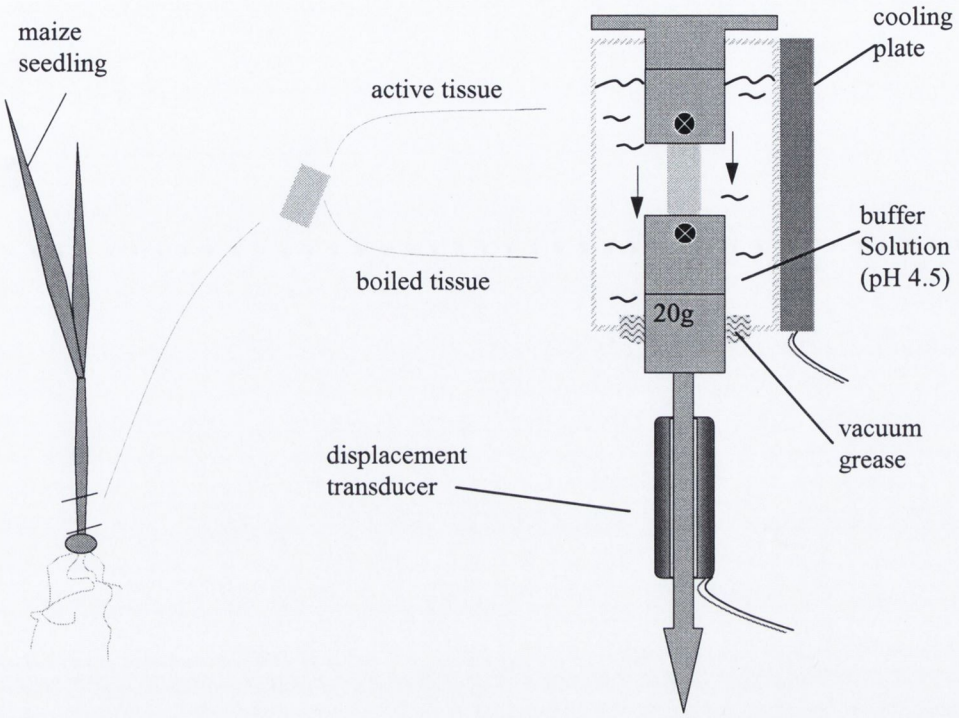


Fig 3.2 Diagram of extensiometer with temperature control. A segment of wall tissue was excised from the leaf extension zone. The segment was frozen, thawed, abraded and then clamped in the extensiometer under a 20 g weight. The extensiometer chamber was filled with a potassium acetate buffer (pH 4.5). Extension of the segments was measured with a displacement transducer attached to the lower clamp. Extension was measured in active tissue and in tissue boiled prior to measurement to inactivate wall proteins. The cooling plate connected to the extensiometer chamber was used to control the temperature of the buffer solution.

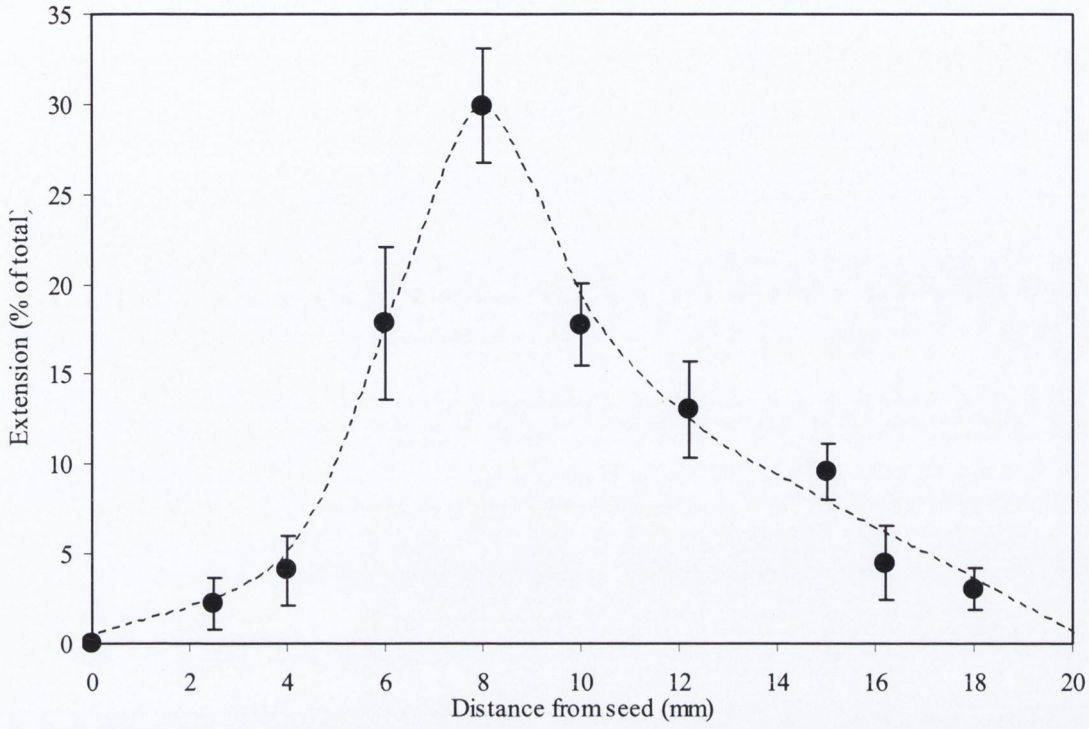


Figure 3.3 Profile of cell extension along the leaf extension zone in *Zea mays* seedlings. Holes were made in the bottom 20 mm of leaf two during extension growth. Values show increased distance between holes expressed as a percentage of total extension after 12 h, bars are s.e. (n=9).

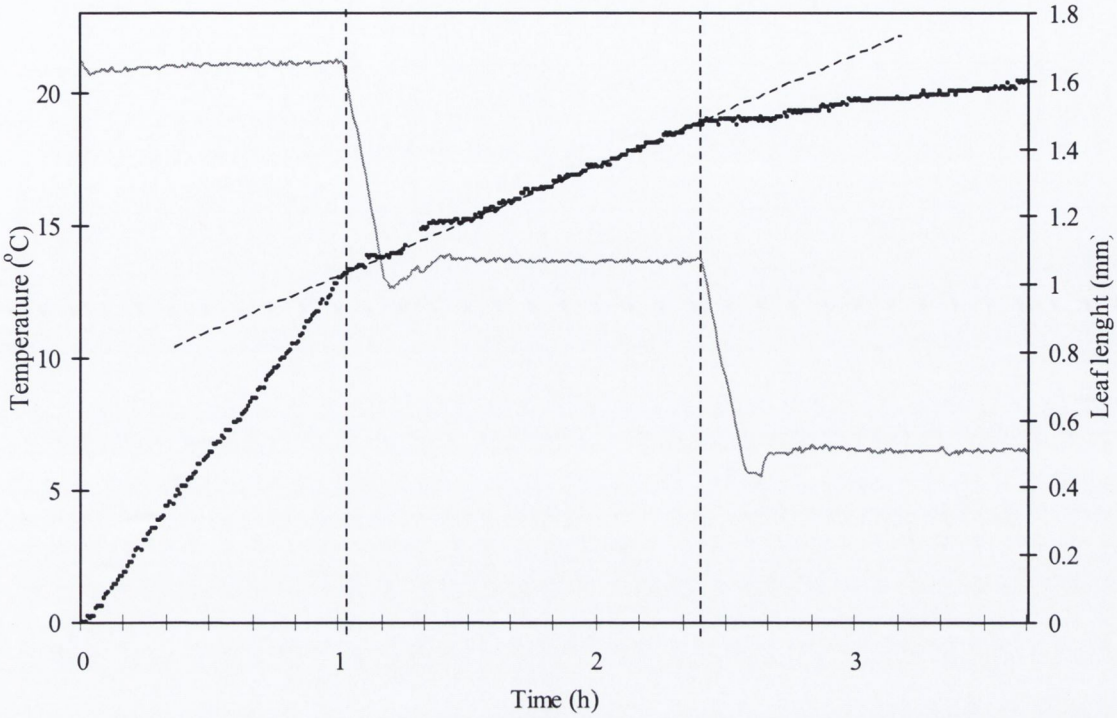


Figure 3.4 The effect of leaf extension zone temperature (grey line) on leaf extension (black squares) in *Zea mays*. Each point is a mean over 30 s. Dashed lines illustrate the rapidity with which leaf extension responds to temperature change.

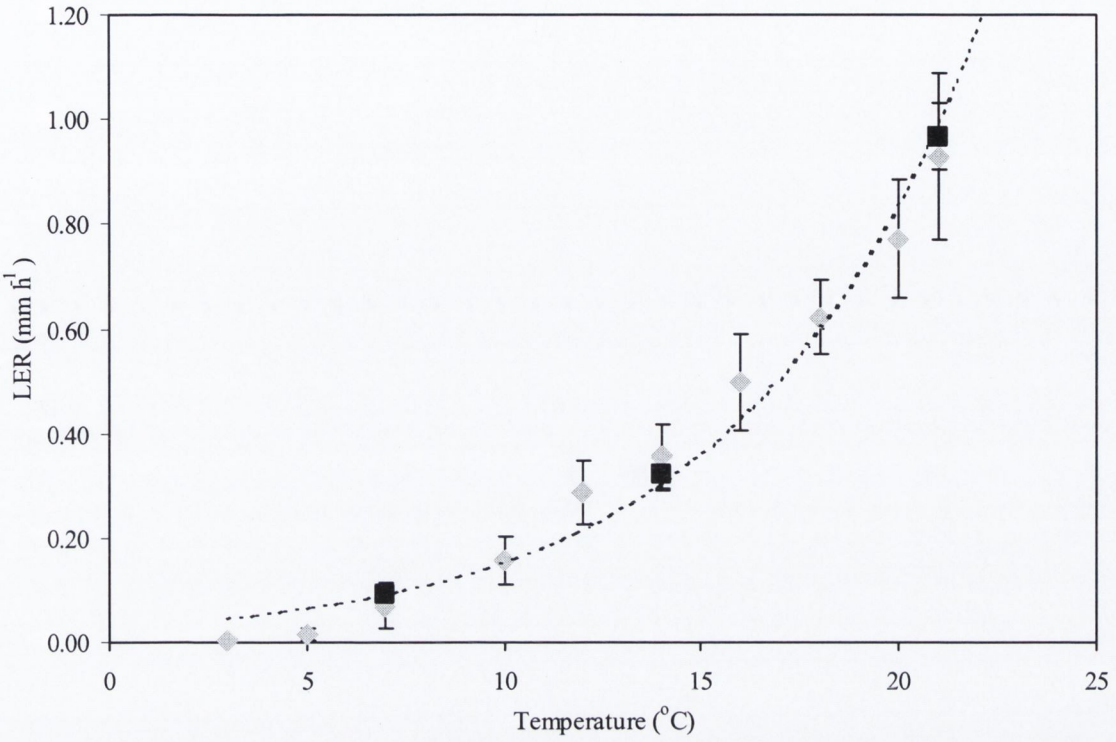


Figure 3.5 The effect of temperature on leaf extension rate (LER) in *Zea mays* (Black squares), bars are s.e. (n=10). Dashed line is an exponential model fitted to the means. Grey diamonds show LER over a wider temperature range, bars are s.e. (n=3).

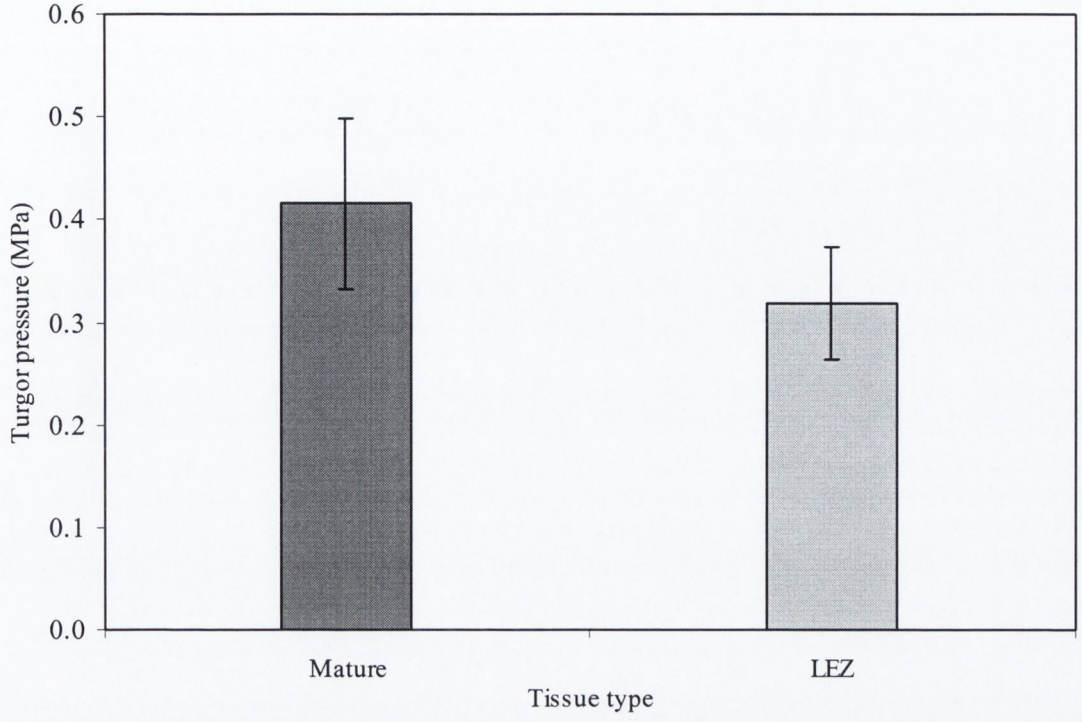


Figure 3.6 Turgor pressure in mature and expanding epidermal cells of *Zea mays* seedlings at 21°C. Data are mean values for 10 cells from 10 seedlings, bars are S.D. Means are significantly different at $P<0.005$, using ANOVA.

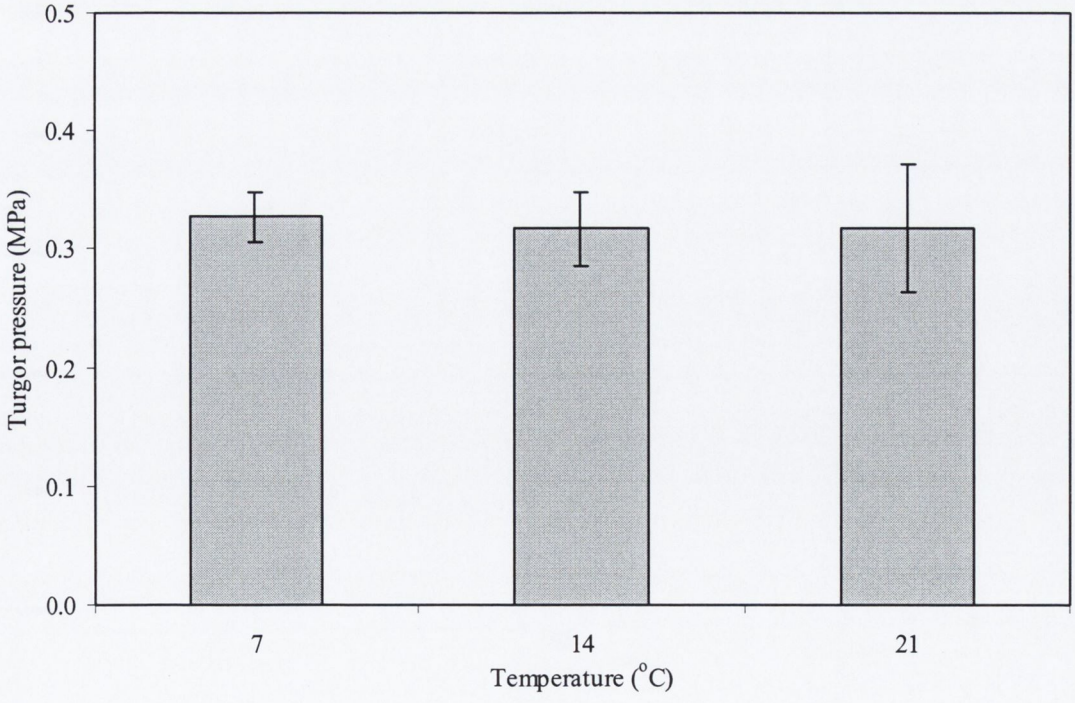


Figure 3.7 Turgor pressure of expanding epidermal cells of maize seedlings, with the leaf extension zone held at 7, 14, and 21 °C, roots and mature leaves kept at 21 °C throughout. Data are mean values for 7-10 cells, measured from 10 seedlings, bars are S.D. No significant difference at $P>0.05$, using ANOVA.

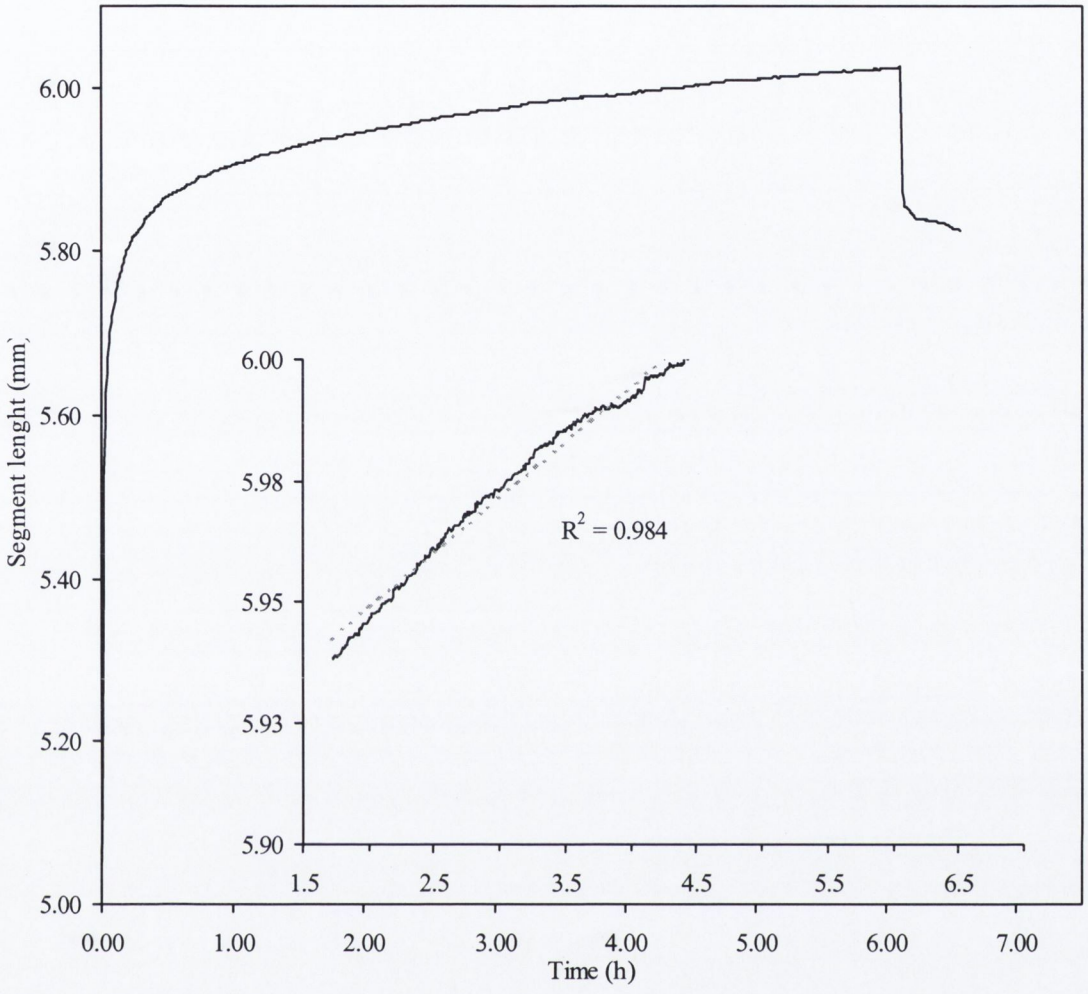


Figure 3.8 Response of segments from the leaf extension zones of *Zea mays* to constant force over 7 h. 10 mm segments of tissue were frozen, abraded, thawed and clamped in an extensiometer (5 mm between clamps) containing 50 mM potassium acetate buffer, pH 4.5. A 20 g weight was applied a time zero and removed after 7 h. Inset shows detail of the response over the time used in subsequent analysis of the thermal response.

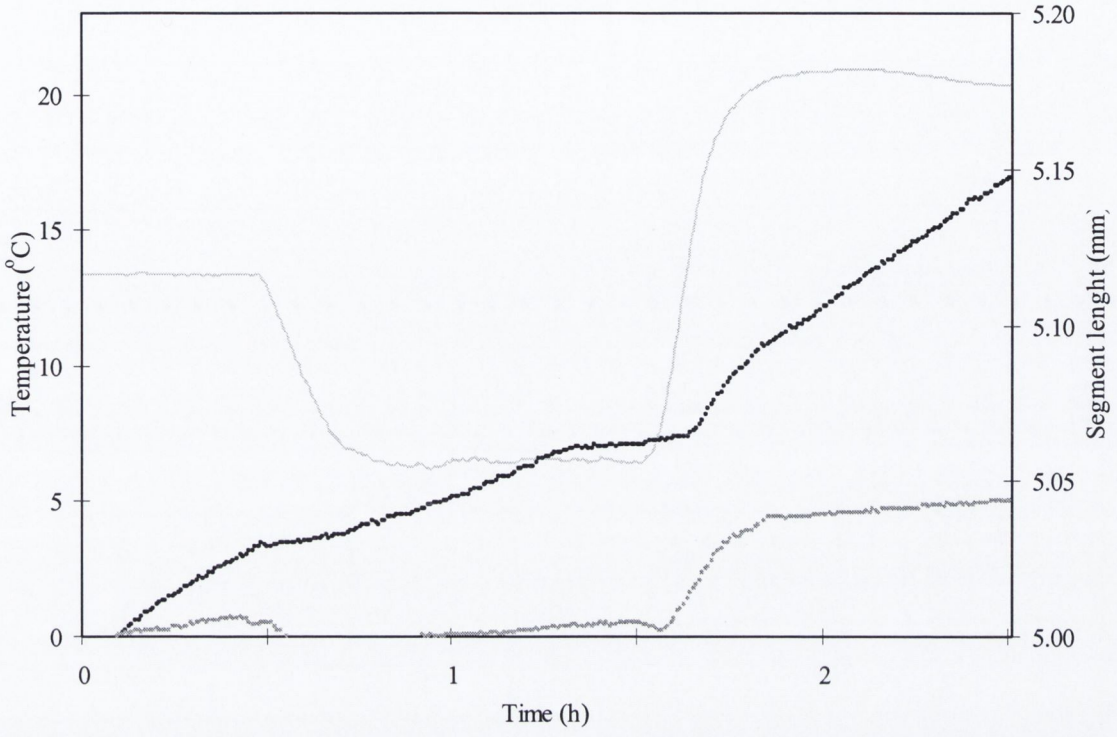


Figure 3.9 Effect of temperature (grey line) on extension of active (black squares) and heat inactivated (grey squares) segments of leaf extension zone tissue from *Zea mays*. Each point is a mean over 30 s.

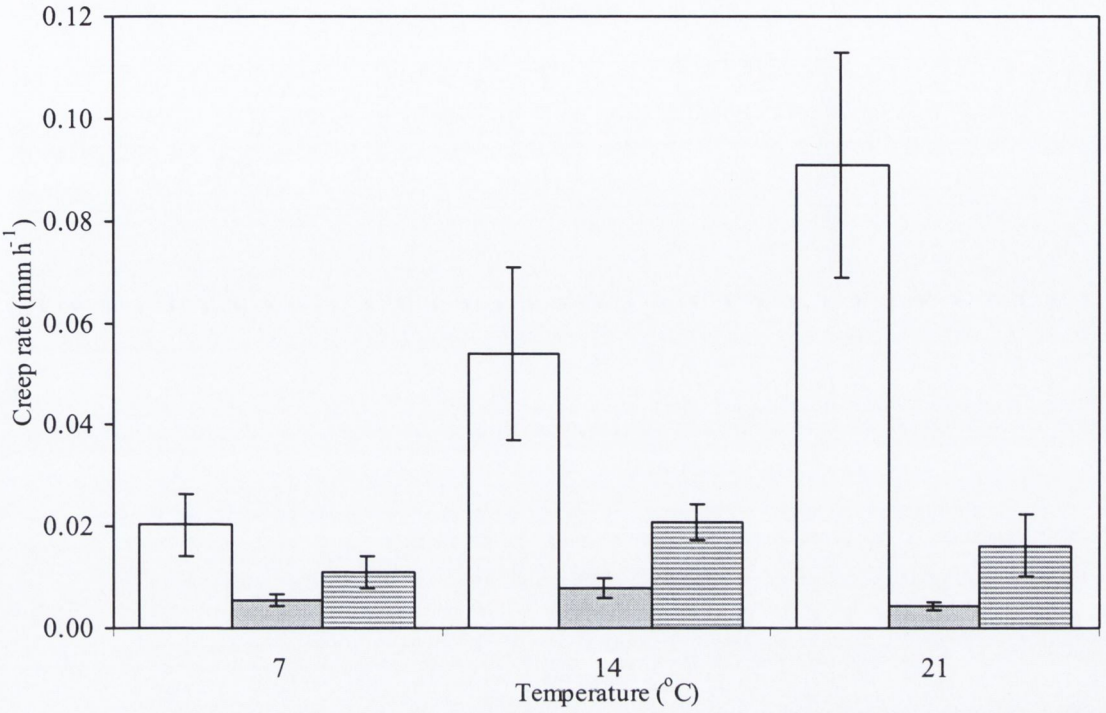


Figure 3.10 Creep rate of segments from the leaf extension zones of *Zea may* at a range of temperatures. White bars: active tissue, grey column: boiled tissue, striped column: tissue measured with 3 M NaCl added to the usual buffer. Bars are s.e. (n=10, 8, and 5 respectively).

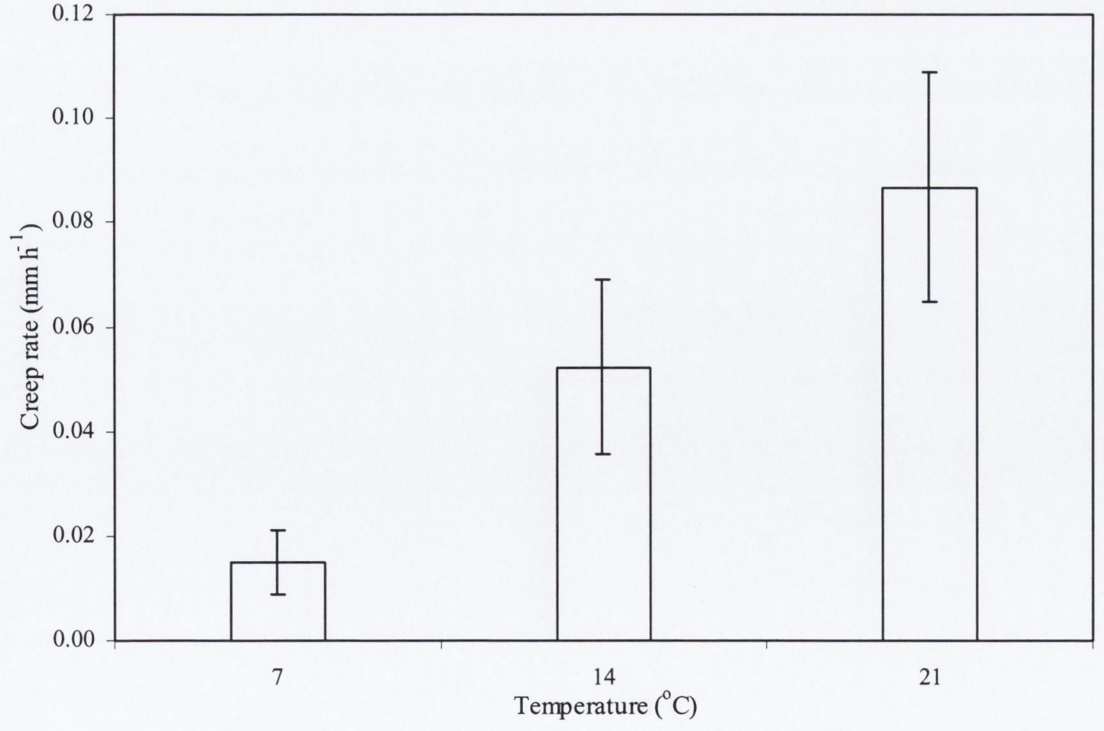


Figure 3.11 Creep rate of segments from the leaf extension zones of *Zea may* at a range of temperatures. Bars show creep rate of active tissue after the creep rate form boiled tissue at the same temperature is removed (see Figure 3.10). Bars are s.e. (n=10).

Chapter 4: *In vivo* measurement of cell wall properties, in grasses with contrasting thermal responses in leaf extension rate

4.1 Introduction

The cell wall plays a vital role in controlling cell expansion. The composition of the wall is influenced by many factors such as tissue type, cell age and genotype characteristics. The growing wall is also subject to constant adjustment by the incorporation of new material and through the regulation of the chemical environment within the wall space (Pritchard, 1994; Cosgrove 1997). Exogenous factors such as temperature will interact with these endogenous controls and thus influence the properties of the wall. To understand the complex nature of such influences it is necessary to look at the wall properties within living tissue.

In this Chapter the effect of cooling on wall properties are investigated *in vivo* with all of the physiological controls in place. A comparison is made between maize, barley, and a *slender* barley mutant. These three grasses show contrasting thermal responses (Figure 4.1), with *slender* barley in particular showing an exceptionally low base temperature for leaf extension. Despite the range of thermal responses theses genotypes are all similar in that turgor pressure in the leaf extension zone (LEZ) is unaffected by short-term cooling (See Chapter 3, and Pollock *et al.*, 1990). The observation that turgor pressure is not reduced during short-term cooling makes it unlikely that water entry is limiting the cell expansion at suboptimal temperatures. Here we focus on the effect of cooling on the cell wall, by utilising the predictions of equation (1.2).

$$RER = \phi(\Psi_p - Y)$$

Where ϕ, the wall extensibility ($mm.mm^{-2}\ MPa^{-1}\ s^{-1}$) determines the rate of irreversible flow of the wall for a given stress, Ψ_p is the turgor pressure (MPa) which provides the stress, and Y is the yield threshold of the wall below which no extension will occur (MPa). In this case RER is estimated from measurements of leaf extension. Equation (1.2) predicts that leaf extension will be a linear function of Ψ_p. This relationship has been found to apply for many isolated cells and intact tissues from several species, though the response is not always linear (Tomos, 1985; Cosgrove, 1993; Pritchard, 1994).

The use of externally applied force to assay the wall properties of living unplasmolysed tissue, has been employed by several authors (Kutschera and Schopfer, 1986; Kutschera and Briggs, 1987; Kutschera and Briggs, 1988; Nonami and Boyer 1990; Cramer and Bowman, 1991; Neumann, 1993; Chazen and Neumann, 1994; Cramer, 2003). The general principle of 'applied force' involves adding a weight to growing tissue to provide extra force in the direction of growth. The methodology varies greatly between studies. The methods used here are most similar to those described in the later half of Cramer and Bowman (1991) with the weight functioning in a 'turgor-like' way increasing the tensile stress in the cell wall and resulting in an accelerated leaf extension rate (LER). Used to its full potential the technique requires the application of a range of forces for a prolonged time (>30 minutes) with each application including an unloading to establish the elastic component of extension. Each application of force yields a value for plastic extension, elastic extension and new steady state LER (see Chapter 1, Box 1.3). Of the studies employing applied force only Cramer (1991) and Cramer (2003) analysed the effect of the extra force on steady state LER with most studies removing the weight after a relatively short period (Kutschera and Schopfer, 1986; Kutschera and Briggs, 1987; Kutschera and Briggs, 1988; Nonami and Boyer 1990; Neumann, 1993; Chazen and Neumann, 1994).

A linear relationship has been found between LER and force applied (Cramer and Bowman, 1991; Cramer, 2003), and between plastic extension and force applied (Kutschera and Schopfer, 1986; Nomami and Boyer, 1990; Cramer and Bowman, 1991; Cramer 2003). The slope of LER by force can be viewed as approximating the relationship between LER and turgor pressure and so acts as a measure of ϕ from Equation (1.2), this parameter is here termed '*in vivo* extensibility'. The slope of plastic extension by force also measures some aspect of wall rheology but given the clear differences between this deformation and the linear extension seen during growth it is perhaps best described as an assay of mechanical compliance (MC). In this sense the latter measure is similar to that measured using the Instron technique, with the advantage that tissue has not been killed, but with the disadvantage that the role of water entry is uncontrolled.

Within living tissue ϕ and mechanical properties are often related, e.g. auxin has been shown to increase both ϕ (Cleland, 1977) and mechanical extensibility (Cleland, 1971b, Kutschera and Schopfer, 1986); while prolonged water stress reduces both ϕ and

mechanical extensibility (Matthews *et al.,* 1984; Nonami and Boyer, 1990). However, it is equally possible that ϕ may be modulated through metabolic controls without altering the bonding arrangements in the wall that determine mechanical extensibility. Several studies have observed changes in ϕ which are not associated with changes in mechanical extensibility, e.g. during exposure to blue-light (Cosgrove, 1988), application of Gibberellin (Cosgrove and Dunford, 1989) and during prolonged salt stress (Cramer, 2003).

Here we utilise the applied force method to investigate the effect of cooling on extensibility and MC of wall tissue in the three contrasting genotypes. The aims are:

1) To test if the inhibition of leaf extension during short-term cooling is associated with a change in *in vivo* extensibility.
2) To ascertain if changes in *in vivo* extensibility are reflected in MC.
3) To identify the source of the variation in LER and thermal response of the three contrasting genotypes.
4) To assess the usefulness of this novel method in identifying genotypic differences in wall characteristics.

4.2 Materials and methods

4.2.1 Maize

A chilling sensitive variety of maize (*Zea mays v. Penjalina*) was used. Seedlings were grown in vermiculite, watered with ½ strength Hoagland's solution (Hoagland and Arnon, 1951), and kept in a growth cabinet (Fitotron, Sanyo, UK) at 21 °C, RH of 65% and a 14 h light period at 300 μmol m^2 s^{-1} Photosynthetic Photon Flux Density (PPFD). All experiments were carried out on the second leaf after 14 days when LER at 21 °C was 0.962 ± 0.042 mm h^{-1} (s.e. n=22).

4.2.2 Barley

Seed stocks of *Hordeum vulgare* L. cv. Herta which are either wildtype or segregating for the *slender* mutation were provided by Chris Pollock at the Institute of Grassland and Environmental Research (Aberystwth, Wales). *Slender* barley plants do not set seed so

plants were obtained form seed of *H. vulgare* Herta Cb3014 which is heterozygous at the *slender* locus and produced progeny segregating 3:1 wildtype to *slender* phenotype. Seeds were imbibed in aerated water overnight then placed on tissue paper suspended above a dish of ½ strength Hoagland's solution. The seeds were germinated and grown in a growth cabinet (conditions as above except temperature at 20 °C) for 7 days. In the wildtype seedlings the primary leaves were 6 –9.5 cm in length, and LER was 0.82 ± 0.15 mm h^{-1}. The *slender* mutants were selected on the basis of leaf length and LER. Those selected were 11.5 –15.5 cm in length, and LER was 1.15 ± 0.13 mm h^{-1}. Selecting *slender* seedlings proved difficult with many batches producing no seedlings with the characteristic phenotype. This may have been due to a lower germination rate among *slender* seeds, a segregation of less than 3:1 or a failure in identifying *slender* plants. The problem was confounded by the fact that only two seedlings could be utilised at a time so any more than 2 *slender* seedlings identified in a batch could not be used. Only 4 seedlings that could be confidently identified as *slender* were produced from 80 (approximately 20 *slender*) seeds available during the experiment.

4.2.3 Leaf extension zone cooling

After 3 days the seedlings were transferred to a specially constructed cooling collar. The cooling collar consisted of a 5 mm diameter copper tube extending for 30 mm to enclose the LEZ. The copper tube was encased in a plastic container through which cooling fluid was passed using a circulating waterbath (F32-HE Julabo, Seelbach, Germany). The temperature of the tube was monitored by a thermocouple placed in the collar adjacent to the seedling, thermal contact was ensured by placing polystyrene foam within the tube to hold the LEZ against the side. The temperature of the mature leaves and soil medium was monitored by thermocouples and remained at the ambient growth chamber temperature throughout. The cooling collar was held in a ridged support grid that prevented the seeds from slipping during applied force experiments.

4.2.4 Leaf extension measurements

LER was measured using auxonometers made from Linear Variable Displacement Transformers (LVDTs) with a stroke length of 100 mm (Type DC/50; Schlumberger, Bognor Regis, UK). These were mounted on weighted retort stands within the growth cabinets. The LVDTs were attached by a clamp to the leaf via a Kevlar thread (Type 989,

440 Dtex; Dupoint, Derry, N. Ireland) that ran over a Teflon pulley. The thread was weighted on the plant side to balance the weight from the LVDT core, an additional 1.5 g was then added to the core to overcome friction. LVDT output was averaged every 2 s, LER was calculated from the slope to leaf length by time using a linear fit ($r^2 > 0.95$).

4.2.5 Applied force

The LVDT was attached to the study leaf one day prior to the experiment. All experimental data was collected at least two hours after the beginning of the light-on period to avoid variation due to physiological adjustment to the light. Once a stable growth rate was achieved an additional elongation force was applied by adding a weight to the LVDT core below the Kevlar thread.

For maize experimental data was collected for 2 h at 21 °C after which the LEZ temperature was stepped from 14 °C to 7 °C and back to 21 °C. Cooling of the LEZ took approx. 15 minutes and each temperature was maintained for 1 h (Figure 4.2). The LER values used are for the final 30 minutes at each temperature. The same procedure was carried out on control plants without applied force.

In barley seedlings the LEZ was cooled to 10 °C for 1 h prior to application of additional force. Once a steady state LER was reached at 10 °C, weights were added in series (5, 10, and 15 g). The weights were kept in place for 2 h so that a new steady state LER could be established (Figure 4.3). The new LER was determined by the slope of a linear regression fitted to the data between 60-120 minutes after weight application. The same procedure was carried out at 20 °C.

The change in leaf length following the application of each weight was divided into a large mechanical deformation that dominates the response immediately after a weight is applied, and a prolonged linear extension that continues until the weight is removed (Figure 4.4). The linear component was determined by fitting a linear regression to the data from 30-90 minutes after weight application. The slope of this line was extrapolated back to zero and subtracted from the original data (Figure 4.4b). The remaining data represents the mechanical deformation produced by the weight. This takes the typical form of extension in viscoelastic materials with an exponential rise to a maximum length (see Chapter 1, Box 1.3). The apex of the curve was used to determine the magnitude of

mechanical deformation. The slope of mechanical deformation by increasing force is a measure of mechanical compliance (MC).

4.3 Results

4.3.1 Maize

Figure 4.5 shows maize LER at each LEZ temperature for control plants and for plants with 20g extra force in the direction of growth. There is a clear thermal response in both seedling types with LER under extra force significantly higher at each temperature. The amount by which LER is increased by the extra force is itself highly sensitive to temperature (Figure 4.6).

4.3.2 Barley

In barley the step increases in force resulted in successively faster LER's, with a linear model providing an adequate fit for control and cooled seedlings. A linear relationship was seen between LER and force applied in both cooled and control seedlings (Figure 4.7). As with the maize seedlings the increase in LER under each weight was less in the cooled seedlings, this resulted in a marked difference in the response of the cooled seedlings to applied force. *In vivo* extensibility was significantly lower in the cooled seedlings; 0.007 ± 0.001 mm h^{-1} g^{-1} compared with 0.025 ± 0.003 mm h^{-1} g^{-1} for the control seedlings. The x-intercept (yield threshold) was remarkably similar between the treatments apparently unaffected by temperature within this range.

The *slender* barley at 20 °C showed a similar response to the wildtype but with higher LER's throughout (Figure 4.8). Yield threshold does not appear to differ between the two genotypes but *in vivo* extensibility was higher in the *slender* seedlings. In Figure 4.9 the *in vivo* extensibility of the three barley types (wildtype, cooled wildtype and *slender*) are shown in relation to the original LER before extra force was applied. There appears to be a linear relationship with an x-intercept close to zero, suggesting that differences in *in vivo* extensibility account for the differences in LER.

The clear differences in *in vivo* extensibility during cooling were not reflected in the mechanical properties of the leaves. Mechanical deformation was seen to increase linearly

with increasing force providing an estimate of MC, however the temperature treatment had no effect on MC or on the x-intercept (Figure 4.10). The *slender* seedlings did show generally larger deformation and a larger slope (MC) than the wildtype (Figure 4.11). However, the slender seedlings showed high variability in deformation making it difficult to draw firm conclusions.

4.4 Discussion

Before discussing the implications of the measurements it is necessary to clarify the meaning of the parameters measured using this novel technique. Most of the studies using applied force have analysed the response over a relatively short timescale and subsequently have not been able to measure the steady state LER under extra force (Kutschera and Schopfer, 1986; Kutschera and Briggs, 1987; Kutschera and Briggs, 1988; Nonami and Boyer 1990; Chazen and Neumann, 1994). Only the data of Cramer and Bowman (1991) and Cramer (2003) provides all of the parameters measured here.

A central concern in using applied force is that if hydraulic conductance (L) is low extension may be partially limited by water uptake into the extending cells. In this case LER is a function of both water transport and wall properties (Cosgrove, 1993). Cramer (2003) overcomes this concern by defining the slope of LER by applied force as $\phi.L/(\phi+L)$, from Equation (1.3). This allows each steady state LER to be partially limited by water transport. However, if L were limiting at any stage the effect would not be an overall reduction in the slope but rather an abrupt loss of response in LER. If at any point water entry failed to keep pace with the externally stimulated LER, turgor pressure within the cell would drop until the original equilibrium was re-established. Such a response would result in constant LER despite the externally applied force. This would cause the slope of LER by applied force to level off as the weight is increased. Such a response is not seen here or in the data presented by Cramer (2003). As such, it seems more appropriated to discuss the slope of LER and force as an estimate of ϕ alone. The results seen here appear to comply to a linear relationship between LER and applied force as predicted by Equation (1.2).

The method used for maize seedlings allows the time scale of the response to be tested. Here we see a rapid response to temperature change similar to that seen for biochemical

creep (Chapter 3). Cooling affects both the original LER and the extra extension (Figure 4.6) with the response equally rapid in both cases. Given that the extra extension is independent of turgor pressure this provides a separate line of evidence illustrating that temperature is acting directly on the cell wall.

In barley seedlings cooling the LEZ has a marked effect on LER. This effect appears to be due to a reduction in *in vivo* extensibility with no change detected in yield threshold (Figure 4.7), or turgor pressure (Pollock *et al.*, 1990), over this temperature range. The change in extensibility occurs without any change in mechanical deformation suggesting that few changes have occurred in wall architecture during cooling. This conclusion is supported by other studies using the Instron technique where no changes in mechanical extensibility were detected during short-term cooling (Thomas, 1990; Pritchard *et al.*, 1990a).

Both the barley and maize data concur with the conclusions of Chapter 3 that temperature is regulated by cell wall properties, with the weight having a greater effect at higher temperatures in all cases. Figure 4.12 shows the maize data presented in terms of the Lockhart equation, assuming a linear relationship between force and LER (as seen in maize over this range of force (Cramer, 2003)). According to this analysis cooling produces a change in both extensibility and yield threshold in the more temperature sensitive maize leaves. This difference in sensitivity of wall properties may partially explain the more marked thermal response seen in maize. An increase in Y was also seen during cooling of maize (Pritchard et al., 1990a) and wheat (Pritchard et al., 1990b) roots; thought in maize the increase was not large enough to be significant and in wheat the response to turgor pressure was not linear making estimation of Y difficult.

The measure of mechanical compliance indicates whether inhibition of leaf extension during cooling was associated with significant alterations in the bonding arrangements in the wall (Cosgrove, 1993). Such changes were found using applied force measurements following auxin treatment (Kustscherea and Schopfer, 1986; Hohl and Schopfer, 1992), and prolonged water stress (Nonami and Boyer, 1990). In this case no such changes were seen, again supporting the conclusions of Chapter 3 that the metabolic component of wall extensibility alone modulates the rate of leaf extension during short-term cooling.

In fact, barley seedlings were cooled for 1 h prior to application of the first weight yet no change was seen in the mechanical deformation compared with un-cooled seedlings. The extent of mechanical deformation remained equal between cooled an uncooled seedlings during the 7 h of cooling used here (Figure 4.10). This suggests no significant changes have occurred in the wall architecture during this time. This is in general agreement with the results for maize roots, where changes in wall architecture only occurred after about 24 h of cooling (Pritchard *et al.,* 1990). Both of these cases provide evidence against a direct link between the mechanical properties of the wall and rate of extension in the immediate past (As discussed in Chapter 3, and Cleland, 1984).

Slender barley seedlings did show a higher *in vivo* extensibility, which appears to account for the original difference in LER (Figure 4.8). The higher extensibility could be due to differences in the functioning of wall loosening factors (as appears to be the case for cooling) or to differences in the bonding arrangements in the cell wall. It was expected that significant differences in wall bonding would be found between wildtype and *slender* barley, as these genotypes show large differences in LER and in thermal response. There is some indication that wall architecture is altered as MC was higher in the *slender* leaves, however, the large error associated with this measure make it difficult to draw firm conclusions. A previous study found that Instron plasticity was almost three times higher in *slender* than in wildtype leaves (Pollock *et al.,* 1990). However, these results are also difficult to interpret as no difference was seen when Instron plasticity measurements were carried out in the conventional way, the difference was only apparent when live tissue was used.

In addition, the results presented thus far do not account for differences in the length of the LEZ. When comparing between plants it is the LER relative to the LEZ length that determines the extensibility of the expanding cells. *Slender* barley seedlings of a similar age to those used here (5 d) have been shown to have a LEZ approximately twice as long as wildtype plants (Schunmann *et al.,* 1994). Table 4.1 shows the values for *in vivo* extensibility and mechanical compliance in the two barley genotypes taking account of the differences in LEZ length. In this analysis not only do *slender* seedlings show a lower MC, the higher LER and *in vivo* extensibility are also lost. This would seem counter intuitive given the higher absolute LERs seen in *slender*. However, an examination of the extension rate of individual cells from each genotype has shown that at no time are

slender cells extending significantly faster than those of wildtype. Instead *slender* cells expand for a longer time following a similar bell shaped pattern in expansion rate eventually resulting in mature cells some 73% longer than those in wildtype (Schunmann *et al.,* 1994). The more elongated bell shaped expansion trajectory followed by the *slender* cells results in a lower relative extension rate overall, but also produces a longer region where cells are extending at near maximum levels. This difference in LEZ length was not accounted for in the comparison of wall properties made by Pollock *et al.,* (1990). The difference they found may be due at least in part to differences in the population of cells tested between genotypes.

The elevated growth seen here in *slender* seedlings appears to be explained entirely by the difference in the LEZ length. The extended period of cell expansion is associated with a change in the mechanical compliance of the LEZ overall. Unfortunately this does not help to explain the ability of this plant to continue cell expansion at extraordinarily low temperatures. It may be the altered bonding arrangements in the wall that allow expansion to continue for longer also reduce the temperature sensitivity of the cells (see Chapter 5, section 5.4.4).

In vivo extensibility as measured by applied force shows good correlation with LER in this case. *In vivo* extensibility alone appears to account for the differences between the three barley leaves tested (Figure 4.9). With the slope between extensibility and LER predicting an intercept near zero, suggesting this parameter has a central role in determining growth. Applied force also appears to provide an estimate to MC in living tissue. However, the usefulness of this method is limited when comparing between plants with different LEZ lengths.

Overall the results point to a controlling role for wall properties during short-term cooling and support the *in vitro* measurements in Chapter 3. Cooling results in significant changes in extensibility without changing the mechanical properties of the wall, indicating the thermal response of leaf extension is determined by the activity of wall loosening factors.

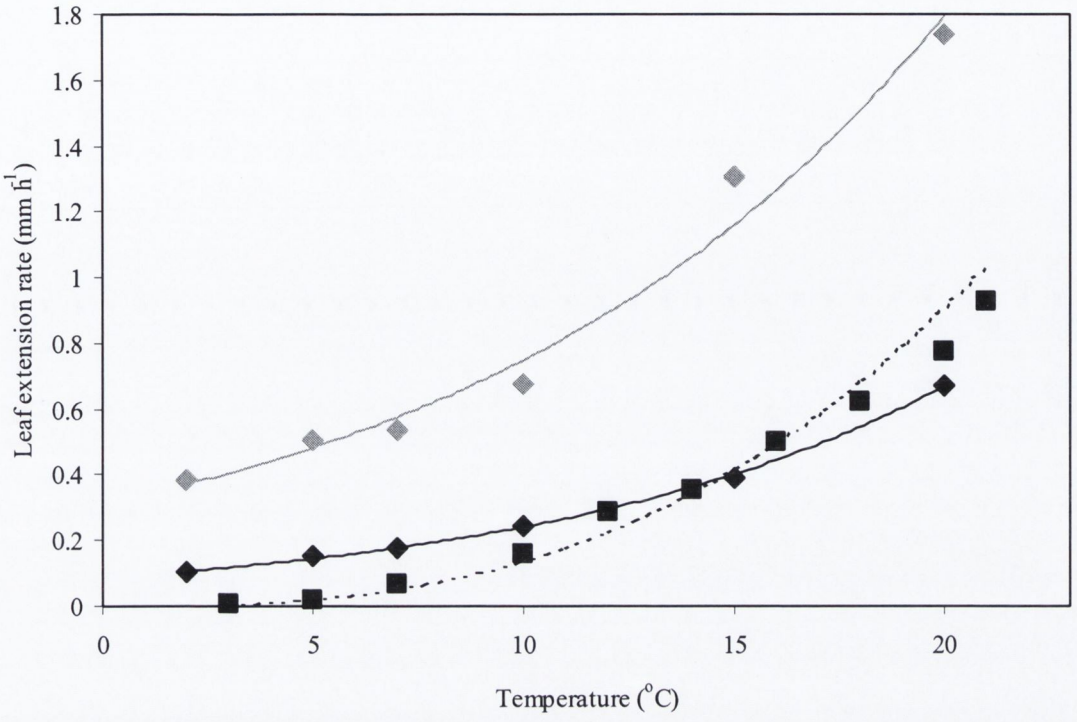


Figure 4.1 Thermal response of *Zea mays* (black squares), *Hordeum vulgare* witld-type (black diamonds) and *H. vulgare slender* (grey diamonds). *Zea mays* and *H. vulgare* show the classic differences between a cold sensitive and a cold tolerant species. *H. vulgare slender* has a novel response showing higher leaf extension rates throughout, a reduced sensitivity to temperature and a lower base temperature. Data from Chapter 3, Figure 3.2 and Pollock *et al.* (1990).

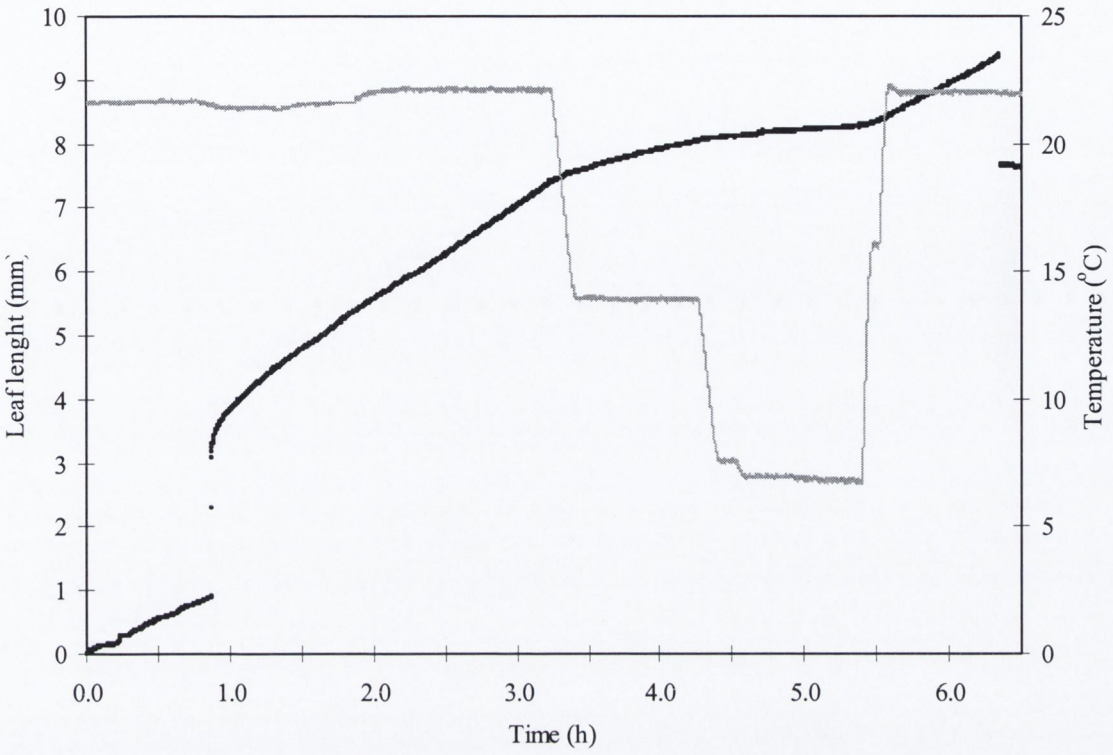


Figure 4.2 Leaf extension of *Zea mays* with extra force applied in the direction of growth. At hour one a 20 g weight was applied to the leaf. Leaf length (black squares) was monitored for 2 h after which temperature around the leaf extension zone (grey line) was changed to 14, 7 and back to 21 °C. At the end of the run the weight was removed.

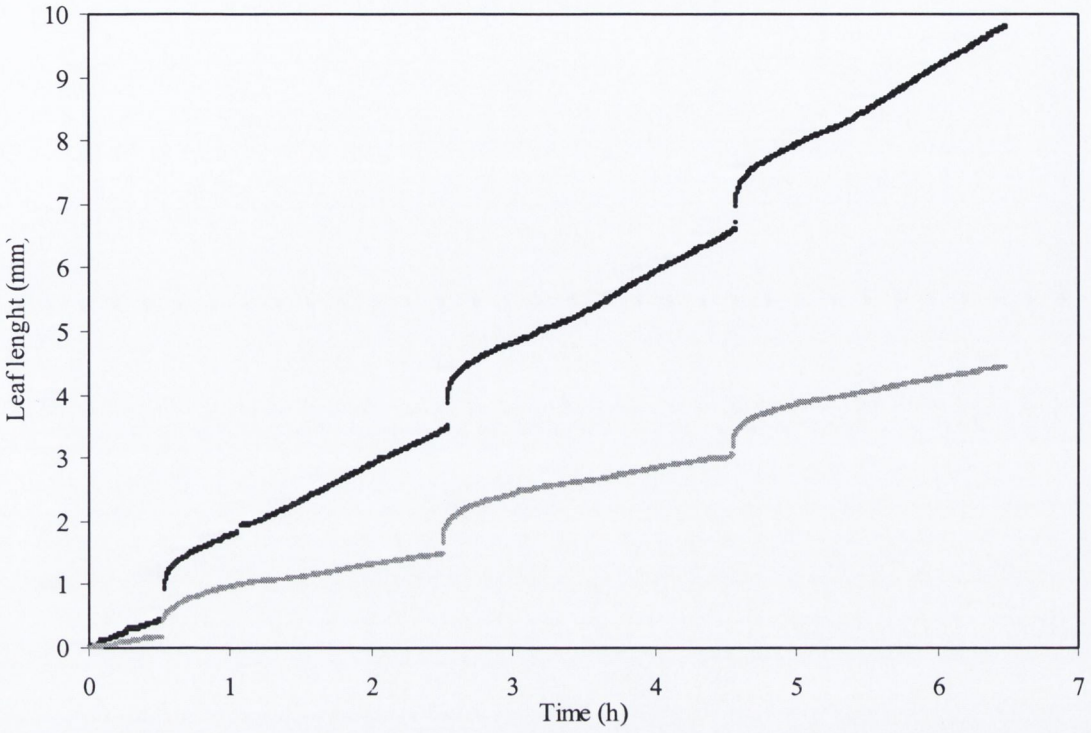


Figure 4.3 Leaf extension of *Hordeum vulgare* during application of increasing force in the direction of growth. Force was increased by adding a 5 g weight every 2 h. Extension is shown for seedlings with the leaf extension zone held at 21 °C (black line), and 10 °C (grey line).

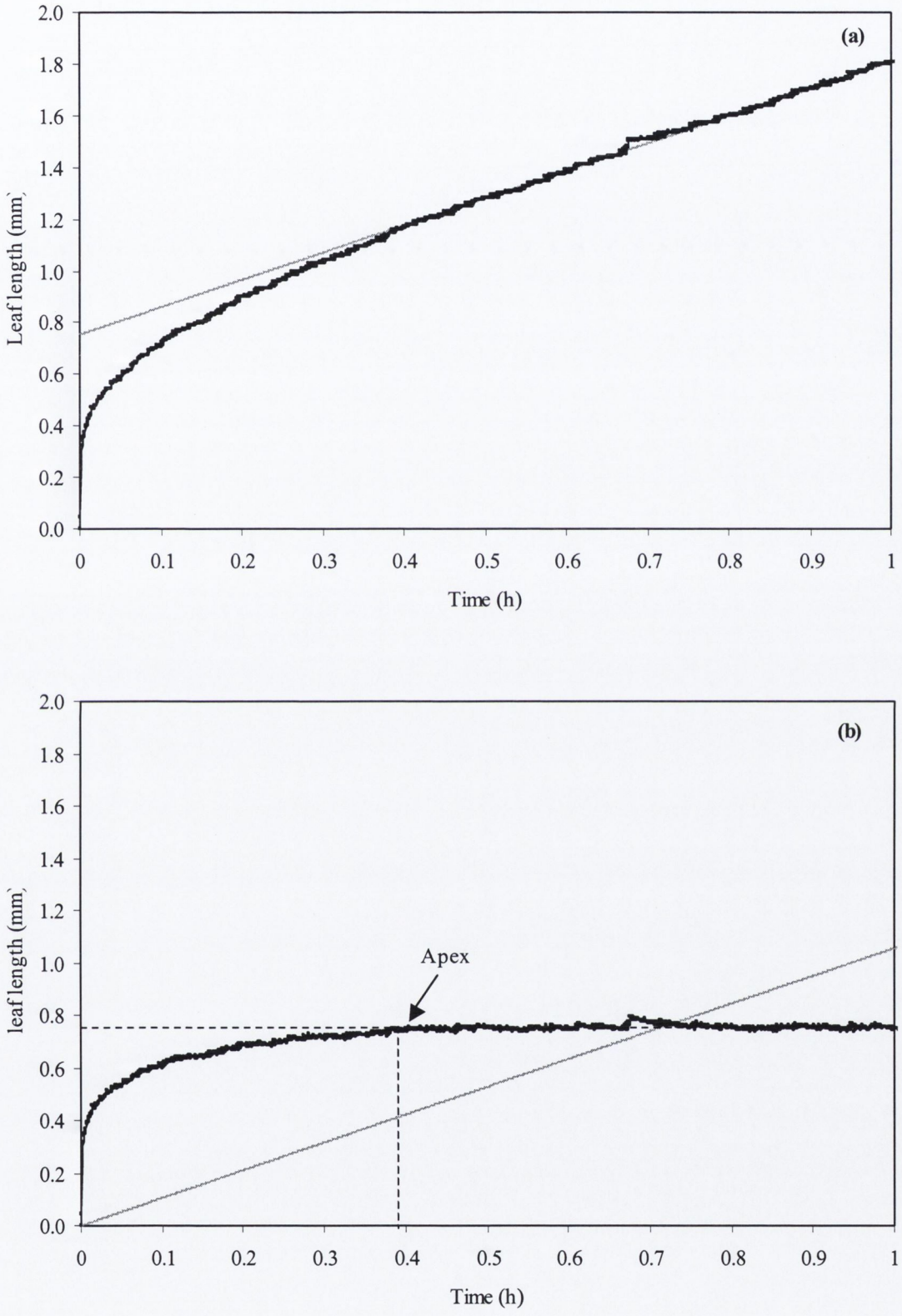


Figure 4.4 Leaf extension of *Hordeum vulgare* during application of 5 g weight in the direction of growth: (a) The overall change in leaf length (black squares), and a linear regression (grey line) fitted to the data from 30 to 90 min; (b) The change in length divided between an extension component which is linear with time (grey line), and a mechanical deformation component which is linear with log time (black squares). The apex of the mechanical deformation curve determines the value and duration of the mechanical deformation.

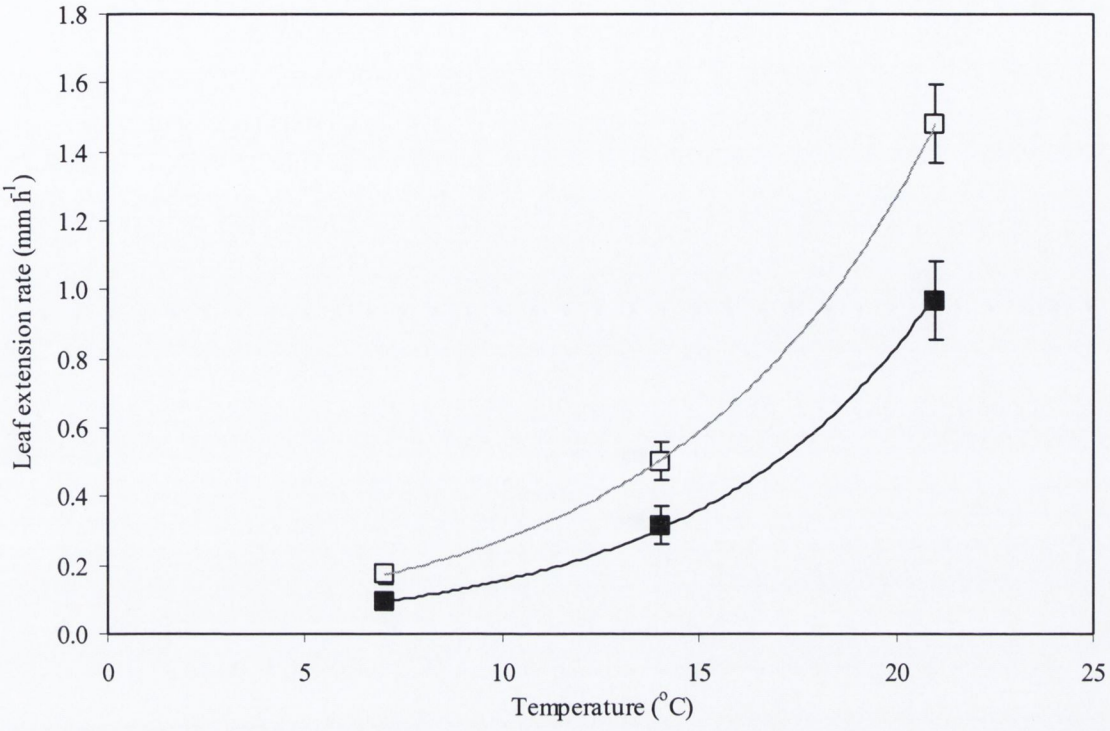


Figure 4.5 The effect of leaf extension zone temperature on leaf extension rate in *Zea mays*. Black squares show seedlings under normal growth conditions, open squares are seedlings with 20 g extra force in the direction of growth. Bars are s.e. (n=11).

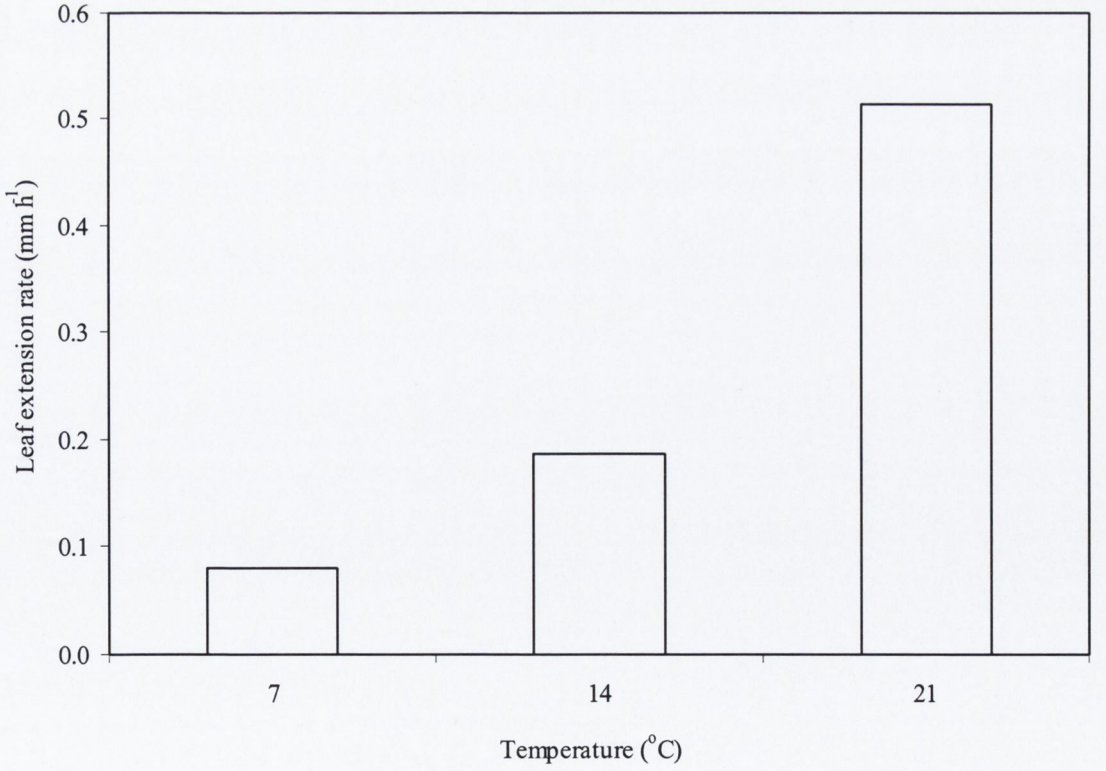


Figure 4.6 The effect of leaf extension zone temperature on the extension rate of *Zea mays*. Columns show the additional extension measured in seedlings with 20 g force applied in the direction of growth, .i.e. the difference between open squares and black squares in Figure 4.5, (n=11).

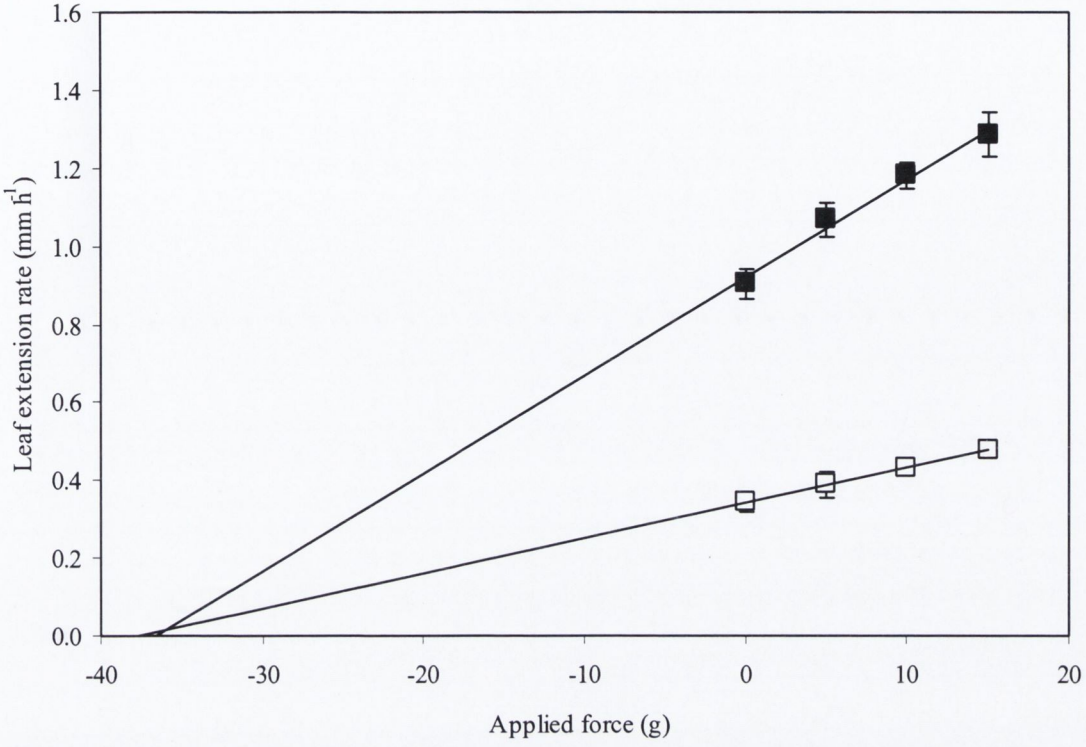


Figure 4.7 The effect of temperature on barley leaf extension rate under different extension forces. Seedlings are held at 20 °C (black squares) or with the leaf extension zone cooled to 10 °C (open squares). Applied force was increased as shown in Figure 4.3. Bars are s.e. (n=6 and 8 respectively).

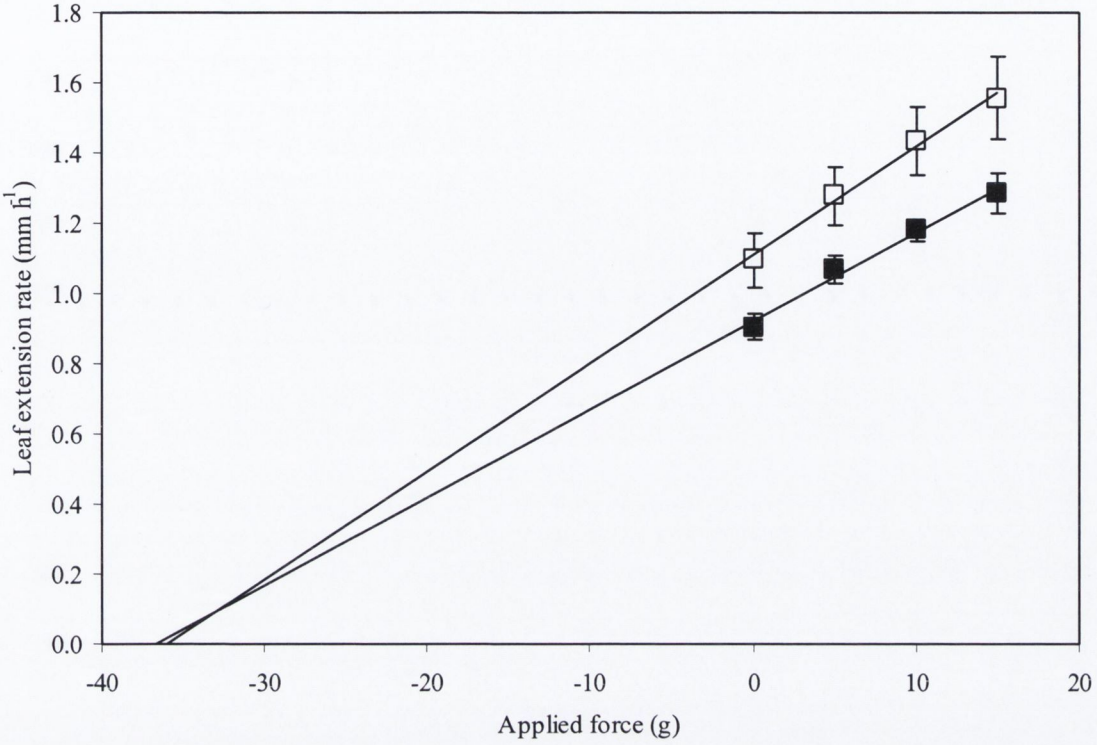


Figure 4.8 Leaf extension rates under different extension forces in two barley genotypes. Genotypes are wildtype (black squares) and *slender* (open squares). Applied force was increased as shown in Figure 4.3. Bars are s.e. (n=6 and 4 respectively).

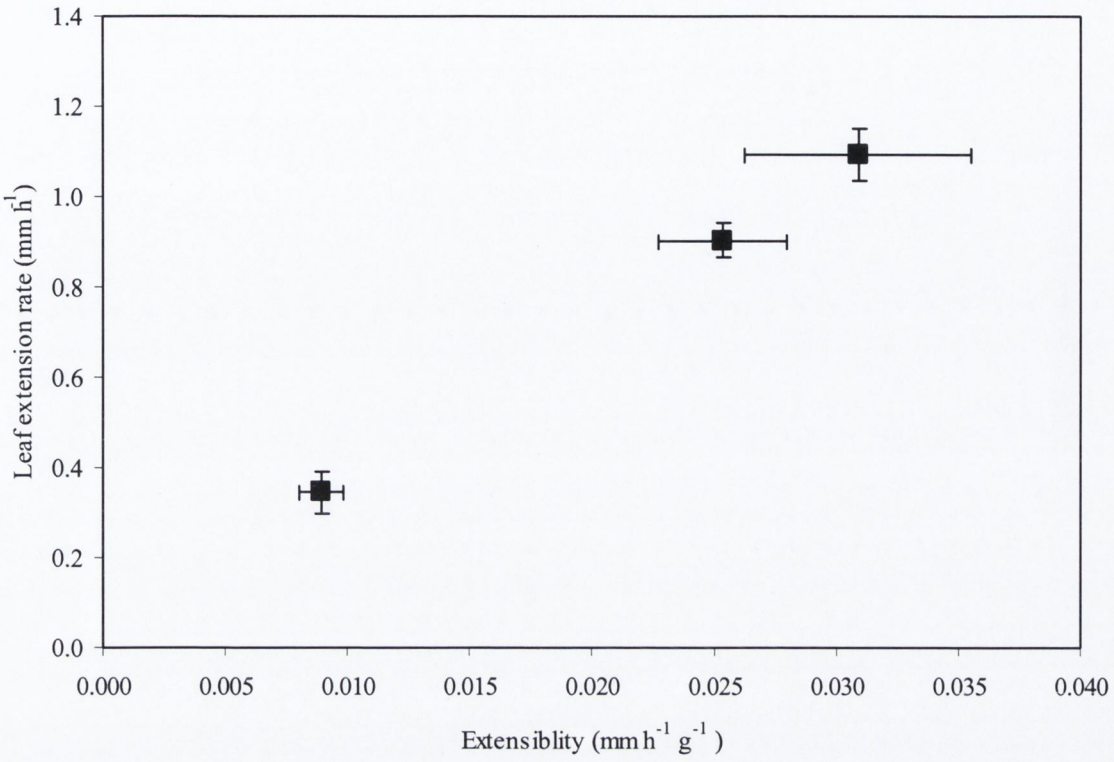


Figure 4.9 The relationship between *in vivo* extensibility and leaf extension rate prior to weight application. Squares show mean values for wildtype barley at 10 °C and 20 °C and for *slender* barley at 20 °C. Bars are s.e. (n =8, 6 and 4 respectively).

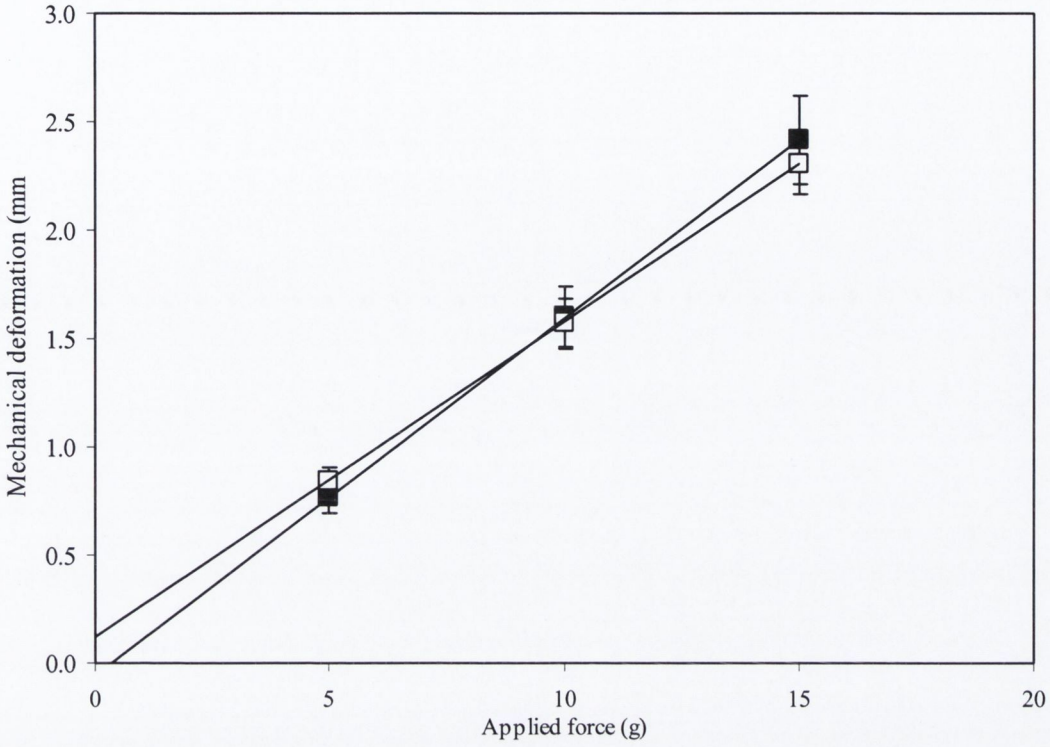


Figure 4.10 The effect of temperature on total mechanical deformation, in barley seedlings with the leaf extension zone held at 20 °C (black squares) and at 10 °C (open squares). Bars are s.e. (n=6 and 8 respectively).

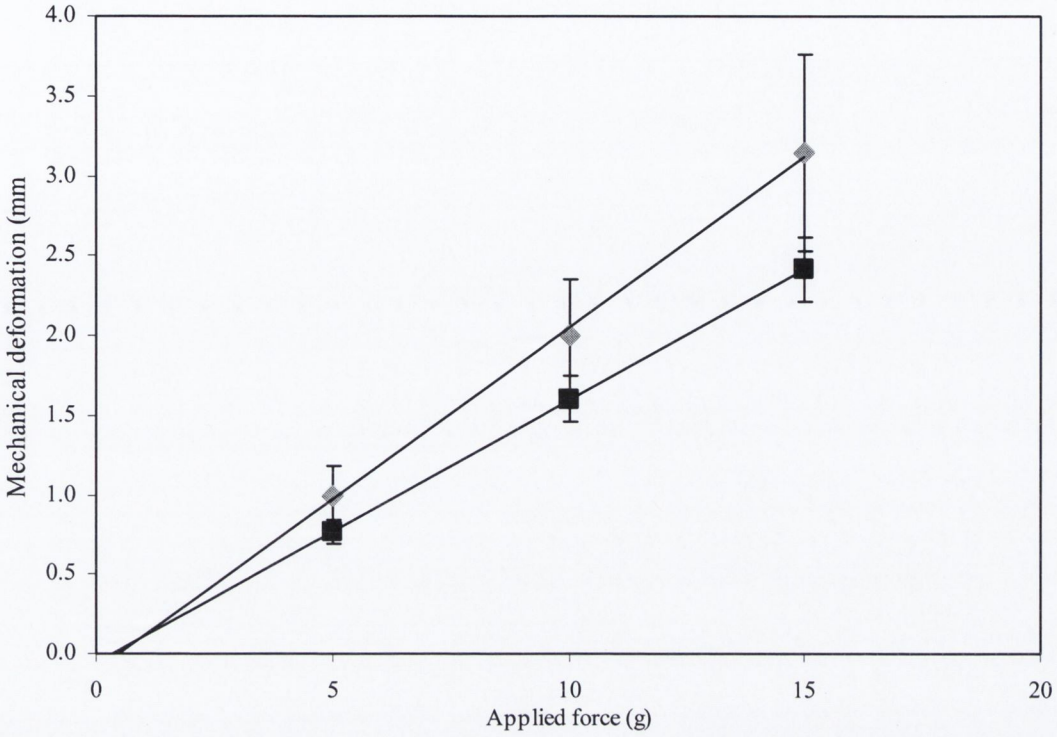


Figure 4.11 The effect of temperature on total mechanical deformation, in two barley genotypes. Genotypes are wildtype (black squares) and *slender* (grey diamonds). Bars are s.e. (n=6 and 4 respectively).

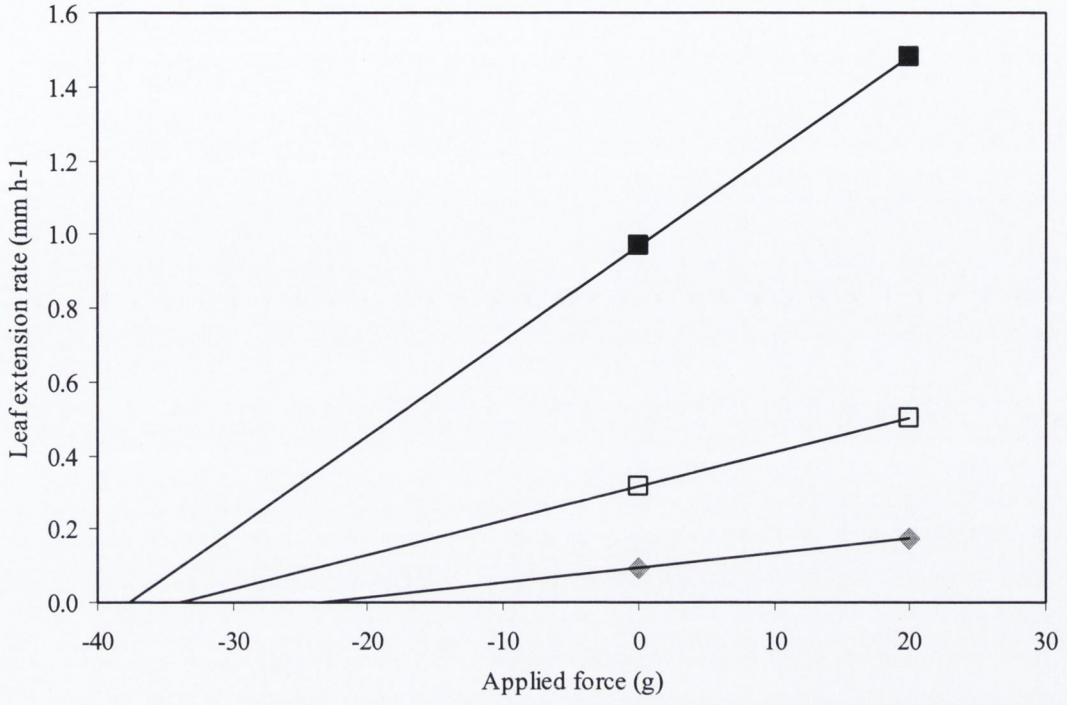


Figure 4.12 The response of leaf extension rate to increasing force in *Zea mays*. With the leaf extension zone held at 7 °C (grey diamonds), 14 °C (open squares), or 21 °C (black squares). Data as in Figure 4.5. The lines are linear regressions representing the extensibility at each temperature as predicted by the Lockhart equation.

Table 4.1 The effect of temperature and the *slender* mutation on growth parameters, expressed relative to the leaf extension zone (LEZ) length. Parameters are relative leaf extension rate (RLER), in vivo extensibility (*m*), and mechanical compliance (MC).

Genotype	Temperature (°C)	LEZ length (cm)*	RLER (cm cm h^{-1})	*m* (cm cm g^{-1} h^{-1})	MC (cm cm g^{-1})
H. vulgare L. cv. Herta (wildtype)	20	1.6	0.057	0.0016	0.103
H. vulgare L. cv. Herta (wildtype)	10	1.6	0.022	0.0006	0.091
H. vulgare L. cv. Herta (slender)	20	2.8	0.039	0.0011	0.077

* from Schunmann *et al.*, 1994

Chapter 5: Final Discussion

5.1 Introduction

There is a well established relationship between radiation interception and crop yield (Biscoe and Gallagher, 1977; Monteith, 1977). Temperature will greatly influence the amount of radiation intercepted by a sward, through its effects on the length of the growing season (Chapter 2) and the rate of leaf extension (Chapters 3 and 4). In this chapter we discuss the mechanisms through which temperature influences theses two factors and the importance of genotypic and phenotypic variation in determining the responses seen.

5.1.1 Main findings of thesis

Chapter 2 examined the influence of temperature on the emergence and survival of four *Miscanthus* genotypes growing in a temperate climate. Shoots of the genotype Sin-H9 had the lowest temperature sensitivity in emergence and the highest frost tolerance. The high frost tolerance in this genotype was associated with significantly lower moisture content in the shoots. Surprisingly this genotype also had the fastest leaf extension rate (LER) at low temperature; a trait often associated with low frost tolerance.

The effect of temperature on LER was further explored in Chapter 3, with maize seedlings showing an exponential decrease in LER when the expanding cells were cooled from 21 to 5 ^{o}C. The restriction of LER was associated with a reduction in the creep rate of cell walls in the expanding zone. The response to temperature was lost in wall tissue that was heat-inactivated prior to cooling, suggesting the reduced cell creep was mediated by wall loosening proteins.

In Chapter 4 the effect of cooling on the cell wall was measured *in-vivo* using maize and barley seedlings. As in Chapter 3 changes in wall extensibility appear to account for the reduction in LER. These changes in wall extensibility occurred without a detectable change in wall mechanical properties. In the temperature sensitive maize seedlings cooling also increased the yield threshold of the wall, while in the more tolerant barley only wall extensibility was affected (in the 10 to 20 ^{o}C range).

In contrast to changes in LER due to cooling, differences in LER between genotypes were associated with differences in both wall extensibility and wall mechanical properties.

5.1.2 Aims of discussion

This final chapter provides a general review of the mechanisms through which low temperature limits leaf emergence and expansion within the context of the findings listed above. It is intended to provide an overview of our current understanding of leaf extension at suboptimal temperatures; across different genotypes and different cooling regimes. In particular it aims to deal with some of the key questions raised by this thesis:

(1) Is there a relationship between leaf frost tolerance and LER at low temperature?

(2) What is the relationship between wall mechanical properties and wall extensibility and why does cooling produce changes in wall mechanical properties in some but not all cases?

(3) How are genotypic differences in the thermal response of leaf extension mediated?

Section 5.2 examines some of the issues raised in Chapter 2 regarding the effect of genotypic differences in leaf emergence and frost tolerance on overall yield.

Section 5.3 provides an outline of the general literature on leaf extension during cooling, and examines the implications of the conclusion that temperature acts directly on wall loosening proteins in this context.

Section 5.4 and 5.5 examine the current state of knowledge regarding the impact of cooling on cell turgor pressure and wall extensibility respectively, again discussing the impact of the conclusions from Chapter 3 and 4.

In Section 5.6 an attempt is made to answer the questions listed above. A model is proposed in which plants with contrasting thermal responses (e.g. different genotypes or plants grown in different thermal environments), display architectural differences in the cell wall which act in conjunction with wall loosening proteins to determine the leaf extension rate at any given temperature.

It is further suggested that the degree to which wall structure will favour rapid extension at low temperature is linked to the degree of frost tolerance in the leaf tissue, and that this

difference is in part determined by gibberellin (or proteins usually associated with gibberellin).

The final section (5.7) provides an overall summary of the thesis along with suggestions for further research.

5.2 Temperature, leaf area and yield

This section examines the role of temperature in determining the length of the growing season and discusses the possibility of achieving yield improvements through breeding for cold tolerance. The low thermal sensitivity of shoot emergence is also discussed as it provides a contrast to the high sensitivity seen in leaf extension.

5.2.1 Temperature and season length the role of breeding

One of the aims of this study was to explore the mechanisms through which low temperature impacts the rate of plant growth by investigating the role of temperature in mediating shoot emergence, leaf extension and seedling survival. In Chapter 2 this aim was explored in the context of cultivating *Miscanthus* genotypes for biomass yield in temperate climates. *Miscanthus* breeding programmes are ongoing throughout Europe with aim of improving overall yield and a specific emphasis on cold tolerance characteristics (Jorgensen *et al.*, 2001; and references therein).

The importance of temperature in determining biomass yield is apparent in the results from the *Miscanthus* production trials in a range of European sites (Chapter 2, Table 2.4). Portugal with a considerably higher average temperature during the growing season had markedly higher yields. For some genotypes yield is twice that of the colder German site. Table 2.4 also illustrates the genotypic variation in thermal response with some genotypes maintaining high yields in the colder sites (i.e. Sin-H10 and Sin-H8), while others fail to grow at all (i.e. Gig-2 and Sac-5).

Those genotypes that yield well in the colder climates must combine the ability to survive temperatures below zero with an ability to maintain leaf extension at moderately low temperatures. As explored in the *Miscanthus* productivity model, in order to produce consistently good yields in frost prone climates several distinct traits are needed. In particular there is a need to combine the ability to produce leaf area early in the season

with an ability to survive spring frosts. To this end it may be necessary to select for several temperature related traits simultaneously. The results of Chapter 2 showed that *Miscanthus* genotypes with the best rhizome frost tolerance did not necessarily have the best frost tolerance in leaves. In fact, across several *Miscanthus* genotypes there appears to be no correlation between the frost tolerances of different tissue types (Jorgensen *et al.*, 2001). In addition, studies with other species have shown that physiological processes such as germination, shoot emergence and growth, are often independently sensitive to cold (Revilla *et al.*, 2000; Tiryaki and Andrews, 2001a, and references therein). This suggests a need to screen separately for many characteristics in order to maximise the length of the growing season and the amount of radiation intercepted during the season.

Breeding programmes with maize have had some success in breeding for low temperature sensitivity in both seedling emergence and leaf growth (Revilla *et al.*, 2000). Combining a high LER and good frost tolerance may be more problematic, as there is often a negative correlation between the rate of leaf extension at suboptimal temperature and frost tolerance (see section 5.3.3). The possibility of such an interaction raises a clear argument against screening for a single trait, such as low temperature sensitivity of LER. However, the negative interaction does not appear to be universal as genotype Sin-H9 with the highest leaf frost tolerance also exhibited the lowest temperature sensitivity in LER. Furthermore, breeding programmes with other species have successfully selected for a combination of good spring growth and cold hardiness (Svenning *et al.*, 1993).

As a relatively new crop *Miscanthus* has the advantage that there is a large gene pool available for use in breeding programmes (Deuter and Abraham, 1998; Scally *et al.*, 2001). The genus *Miscanthus* has a wide distribution in Asia, extending from the subtropical to the subartic (Numata, 1969; Numata, 1974; Hodkinson *et al*, 1997; Hayashi *et al.*, 1981). The distribution also covers a considerable altitudinal range, extending from sea level to more than 3000 m (Weng and Ueng, 1997; Hodkinson *et al*, 1997). This includes a *Miscanthus* population at 3280 m in Papua New Guinea, which appears to exhibit cold-tolerant traits (Earnshaw *et al.*, 1990). These wild populations may yet prove invaluable in extending the range of *Miscanthus* into cooler and more extreme climates.

For the *Miscanthus* genotypes that have already been incorporated into breeding programmes there is a considerable store of information now available, describing genotypic variation in growth characteristics such as, winter frost tolerance (Clifton-

Brown and Lewandowski, 2000), T_b (Chapter 2), Spring Frost tolerance (Chapter 2), LER (Chapter 2; Clifton-Brown and Jones, 1997) and thermal response of LER (Chapter 2; Clifton-Brown and Jones, 1997). It is hoped that this information will aid the selection of new genotypes suitable for the range of climates across Europe and elsewhere.

5.2.2 Selecting for early emergence

The thermal response of shoot emergence (or seed germination) provides an interesting contrast to that of leaf extension. In Chapter 2 the thermal requirement for emergence was represented as DD_{emerge}, i.e. the number of days to emergence times the growth temperature above T_b. This parameter does not appear to have been used previously in the literature. It provides a useful quantitative variable for comparing between genotypes and has the advantage that an independent value for DD_{emerge} is produced at each growth temperature (as distinct from the thermal coefficient and T_b, which are derived from the whole data set). The significant genotypic variation in this parameter (Chapter 2, Table 2.2) suggest it will be useful in future studies screening for emergence and germination characteristics.

The rational for calculating DD_{emerge} is an assumption of a linear relationship between emergence rate and temperature above T_b. A linear response is clear in many germination studies across many species (Angus *et al.*, 1981; Roberts, 1988; Steinmaus *et al.*, 2000). As pointed out by Roberts (1988), an exponential response to increasing temperature would be expected for a metabolically driven process. So, it is not at all clear why there should be a linear relationship between and rate of germination and temperature. One explanation is that the underlying response is exponential but appears approximately linear over a limited temperature range. If this were the case we would expect to see DD_{emerge} fall as growth temperature is increased. No such pattern is apparent in the *Miscanthus* genotypes tested here or in the data presented in other studies, even where the response was measured throughout the range from T_b to optimal temperature (Angus *et al.*, 1981; Roberts, 1988; Steinmaus *et al.*, 2000). An alternative explanation is that because the initiation of germination is dependent on a hormonal signal rather than a set of biochemical reactions the response is only indirectly responsive to temperature. It may be that hormone accumulation (gibberellin in this case (Davis, 2002)) shows the expected exponential thermal response but that the response of germination to hormone concentration is not linear. Many development processes have been shown to have a

logarithmic rather than linear response to the concentration of hormones (Roberts, 1988). This may represent a fundamental difference between processes which are directly reliant on the rate of biochemical reactions and so are highly temperature sensitive e.g. leaf extension, and processes which are modulated by hormonal signals from within the plant and are only indirectly reliant on temperature, e.g. germination. This distinction may be important in identifying rate-limiting processes within plants growing in suboptimal climates, and in identifying the extent to which in may be possible to reduce the temperature sensitivity of different processes through breeding or application of growth regulators.

5.3 Temperature and leaf extension

This section provides an outline of the general literature on leaf extension during cooling, and examines the implications of the conclusion from Chapter 3 that temperature acts directly on wall loosening proteins. In particular there is an assessment of the relationship between LER at suboptimal temperatures and leaf frost tolerance, and of the possibility that acclimation of LER occurs during prolonged cooling.

5.3.1 The thermal response

Unlike germination, the response of maize LER to short-term cooling clearly includes the expected exponential response. However, the fit is imperfect, as it will tend to underestimate the base temperature where extension stops and overestimate the higher LER values (Figure 3.1). The response appears to be better represented as an Arrhenius plot Figure 5.1.

The linearity of the Arrhenius plot is based on the assumption that a 10 ^{o}C increase in temperature doubles the number of molecules that have a sufficiently high activation energy to undergo reactions (Sutcliff, 1977). Expressed in this way the data conforms to a biphasic model seen in the thermal response of many physiological processes (Sutcliff, 1977; Miedema, 1982; Nobel, 1988; Clarkson *et al.*, 1988). Above 10 ^{o}C the activation energy of 103 kJ mole^{-1} is typical of metabolically regulated plant processes. Many reactions of importance in biochemistry have activation energies of 50-100 kJ mole^{-1}, which leads to Q_{10} values of 2-4 (Nobel, 1988). Within the current model for wall

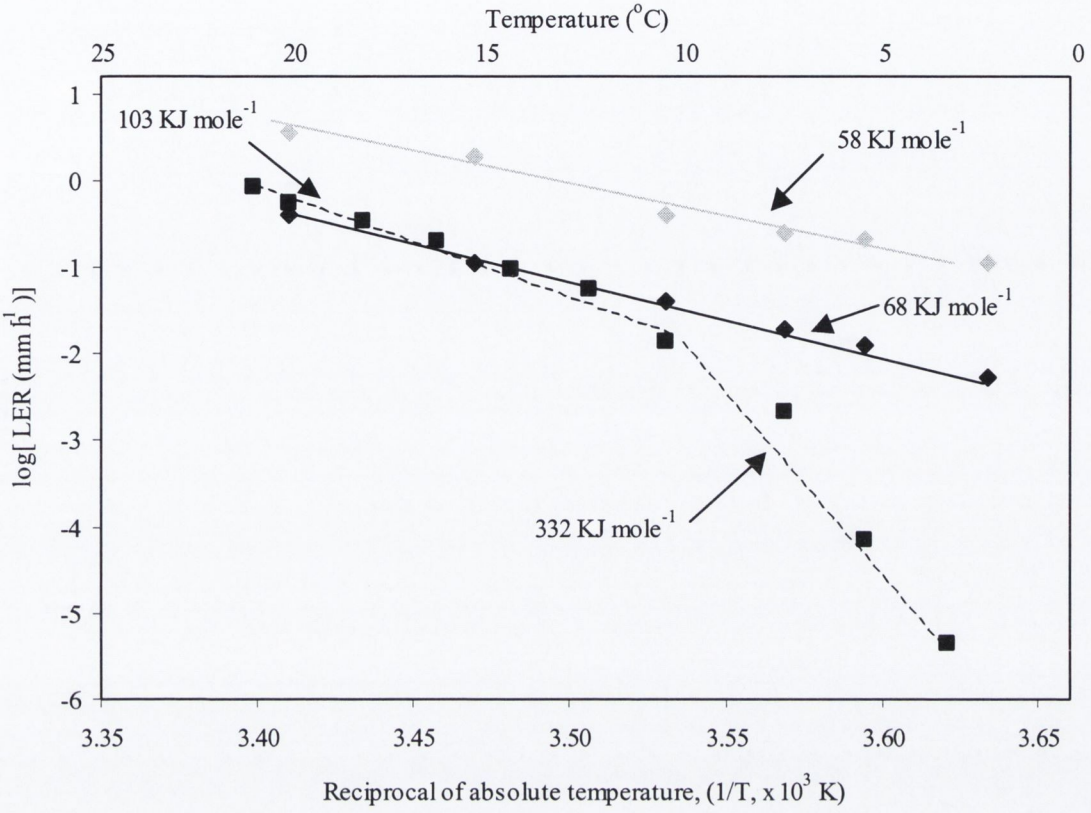


Figure 5.1 Arrhenius plots of leaf extension rate (LER) in maize (black squares), wildtype barley (black diamonds) and *slender* barley (grey diamonds). Activation energies are shown (calculated as: [-1 x temperature coefficient] x the universal gas constant). Maize and barley show the classic differences of a cold sensitive and a cold tolerant species. Data as in Figure 4.1.

loosening, this activation energy represents the energy required to break a series of hydrogen bonds that tether the cross-linking glycans to the surface of cellulose microfibrils (see Chapter 1, section 2.5; McQueen Mason and Cosgrove, 1993; Cosgrove 2000).

The change in slope below the inflection point (10 °C in this case) is generally thought to be associated with a phase transition in membrane lipids. Cooling produces changes in the spatial organisation of membrane lipids, that have been shown to result in decreased fluidity leading to changes in the activation energy of membrane enzymes (Levitt, 1980; Miedema, 1982; Nobel, 1988; Clarkson *et al.*, 1988; Somerville *et al.*, 2000). Below the inflection point the thermal response of LER is much higher with an activation energy of 332 kJ $mole^{-1}$, reflecting the importance of the phase change in determining leaf extension in the lower temperature range.

It is unclear what effect membrane fluidity would have on the activity of enzymes in the wall itself, though clearly the plasma membrane and cell wall are closely associated. It may be envisaged that alteration in some of the ephemeral wall properties such as hydration of the wall matrix or H^+ efflux could result in the biphasic response seen here (see section 5.5.1). Chilling resistant and chilling acclimated plants often have a higher proportion of unsaturated lipids, which has the effect of lowering the temperature at which membrane phase transitions takes place (Williams *et al.*, 1988; Palta *et al.*, 1993; Bray *et al.*, 2000). The importance of membrane lipids in the thermal response has also been demonstrated in mutant plants where the proportion of unsaturated lipids was intentionally altered (Wu *et al.*, 1997). These mutants were investigated in terms of photosynthetic thermal response, but could also provide a system for testing the degree to which this aspect influences LER in the lower temperature range.

Alternatively the appearance of the biphasic response for leaf extension may result from a phase change in the wall itself. The hydrated gel matrix formed by pectins and other wall constituents (Carpita, 1996) could itself undergo phase transitions similar to that experienced in the plasma membrane. It has been suggested that pectins can limit extension through their effect on wall porosity and thus the access of expansins to their glycan substrates (Carpita and McCann, 2000).

The barley plants appear considerably more chilling resistant with an activation energy of 68 kJ $mole^{-1}$, and no clear change in slope down to 2 °C (Figure 5.1). This may represent a difference in the response of membrane lipids or it may reflect a more lasting difference in the bonding arrangements in the wall (see section 5.5).

The *Miscanthus* genotypes investigated in Chapter 2 also showed variation it the thermal response of leaf extension with Sin-H9 exhibiting the low sensitivity typical of cold adapted ecotypes. Such variation may be crucial in understanding further the nature of the response of leaf extension to cooling. In discussing this response, it is important to distinguish again between long and short-term cooling and to consider the relative importance of evolutionary adaptation, previous acclimation and the interaction between the two.

5.3.2 Adaptation

Woodward (1975) found that *Sedum* species adapted to grow in cold upland habitats have a reduced temperature sensitivity allowing them to out compete (out-grow) their lowland relatives. When the plants were transferred to higher temperatures (above 10 °C) the situation is reversed as the lowland *Sedum* species show a much greater increase in leaf area. A similar situation was seen in *Poa* with upland ecotypes having slower LER at higher temperatures but a reduce temperature sensitivity allowing higher LER below 10 °C (See Figure 1.1; Korner and Larcher, 1988). This represents the classic cold adapted behaviour from an ecological perspective. As discussed in Chapter 3 the contrasting responses of the two ecotypes is due, at least partly, to differences in wall structure (Woodward and Friend, 1988).

A very different perspective is seen in the literature focused on cultivated plants. Pollock and Eagles (1988) reviewed a series of experiments comparing the response to chilling in forage grasses from Northern European with relatives from warmer southern regions. Many aspects of plant morphology changed in response to chilling and in each case the responses were more marked in the northern populations (Cooper, 1964; Robson and Jewiss, 1968a,b; Ostgard and Eagles 1971b; Eagles and Othman, 1981). Leaf extension in particular was found to be more sensitive to chilling (over several weeks) in the northern ecotypes. Thomas and Stoddart (1995), carried out a detailed study of the thermal responses in closely related *Festuca arundinacea* Schreb. and *Dactaylis glomerata* L.

ecotypes, with a range of geographical origins. They also conclude that, "in general, ecotypes from the coldest habitats were the most sensitive to low temperature inhibition".

It appears that two distinct adaptive strategies have developed in different cold regimes: plants from high altitudes have adapted in a way that reduces the inhibition of cell expansion during cooling, while plants from northern regions have adapted by developing increased inhibition of cell expansion during cooling. It is at first surprising that northern ecotypes would be more sensitive to cooling however as suggested by Ostgard and Eagles, (1971b), this probably represents an adaptation promoting winter survival with the plants becoming dormant as temperatures fall.

Does lowering LER at suboptimal temperatures increase frost tolerance?

Several studies have found a strong negative correlation between growth rate at low temperature and frost tolerance (Cooper, 1964; Humphreys, 1989; Junttila *et al.*, 1990; Pollock *et al.*, 1993 Svenning *et al.*, 1997; Rapacz, 1998). This is presumably related to the strategy of developing winter dormancy as found in the northern ecotypes discussed by Pollock *et al.* (1993). As discussed above, such a strategy may not always be the most effective in ensuring survival in cool climates. Rapacz, (1998) presents the dilemma as follows: "It has been suggested that the first stage of frost hardening of herbaceous plants is related to the cessation of growth. Inhibition of cell expansion brings about decreased tissue hydration. This may in turn limit the probability of intercellular freezing... On the other hand, the cessation of growth in terms of dry matter accumulation may reflect the worse energy supply caused by low net photosynthesis. Thus, plants that grow faster are more resistant".

The results of Chapters 3 and 4, show the reduced leaf extension during cooling can be explained in terms of direct temperature effects on wall extension rather than a restriction of carbon supply. The same appears to be true during long-term cooling (see Chapter 1, section 1.1). Given this we may assume that Rapacz's first proposal is true, i.e. reducing LER at suboptimal temperatures is related to the development of frost tolerance. Studies of changes in the cell wall during cooling also seem to support this proposition (see section 5.5.3).

However, this relationship is not universal; in Chapter 2 the *Miscanthus* genotype Sin-H9 with the highest tolerance to frost also showed the lowest temperature sensitivity in LER.

The response of Sin-H9 appears to have more in common with that of the upland ecotypes studied by Korner and Larcher (1988), and Woodward (1988). In this case the strategy may be aimed at optimising the production of leaf area in order to maximise radiation capture during a short growing season.

Why do upland species show the opposite response to northern species? The short answer may be that they don't. The thermal response shown in Figure 1.1, represent the LER during the peak Alpine growing season, which lasts for about 2 months. For much of the year the upland plants do reduce LER and biomass production, to zero (Korner and Larcher, 1988). However, because night frosts will occur throughout the year including the growing season, upland plants must combine the ability to expand leaves rapidly with the ability to withstand frost. Such plants presumably rely on frost resistance mechanisms that do not require a reduction in leaf extension rate, such as high solute content or anatomical adaptations (see Box 1.1).

In fact although reducing cell expansion may aid the reduction of moisture content in many cases, some plants may be able to utilise the low moisture content mechanism without reducing LER. For example in Chapter 2 Sin-H9 showed both the highest LER and the lowest moisture content of the four *Miscanthus* genotypes.

The realisation that there are two separate gradients in the physiological response to cooling – one running north to south, the other upland to lowland, opens the possibility of exploiting a matrix to agronomically useful characteristics. The ideal genotype for a particular field site will depend on the pattern of frosts and the duration of the growing season for which temperatures are suboptimal. Where no frosts occur during the growing season the southern lowland type will make best use of the high temperatures; where frosts occur mostly at the end of the growing season the northern plant type would be favoured; where frosts are unpredictable and may occur throughout the growing season the upland strategy will provide the best response even though some yield will be lost due to slow growth during warm periods.

5.3.4 Acclimation

The leaf extension responses discussed above were measured either in the field or during long-term chilling. As such, the characteristics seen result from the combined influence of

adaptation and acclimation. A large number of morphological and physiological changes are associated with acclimation to chilling and the development of frost tolerance (see Box 1.1). Here we are concerned only with the acclimation of leaf extension. In particular, we examine the possibility that plants adjust their LER to suit the prevailing temperatures as occurs during acclimation of photosynthesis (Berry and Bjorkman, 1980; Fitter and Hay, 2002).

Despite the 'avalanche' of information on cold acclimation in relation to frost tolerance (Guy, 1990), there appears to be few cases where acclimation of leaf extension has been considered. Many authors have measured the response of leaf extension to cooling over short periods (<2h) (Watts, 1972a; Peacock, 1975a; Gallagher, 1976; Gallagher, 1979a, c; Thomas and Norris, 1977; Stoddart and lloyd, 1986; Korner and Woodward, 1988; Woodward, 1988; Thomas, 1989; Pollock, 1990; Clifton-Brown and Jones, 1996, Lafarge *et al.*, 1998) or over long periods (Woodward, 1975; Woodward, 1979, Miedema, 1982; Thomas, 1983; Woodward and Friend, 1988; Bos and Neuteboom, 1998; Bos *et al.*, 2000). In some case LER during short and long-term chilling has been measured in the same species (Kleinendorst and Brouwer, 1972; Thomas and Stoddart, 1995; Ben Haj Salah and Tardieu, 1996; Clifton-Brown, 1997). However, few studies have monitored the leaf extension during the transition from a short to a long-term response (Kleinendorst and Brouwer, 1972; Thomas and Stoddart, 1995; Thomas, 1983) and fewer still have specifically examined the LER during the transition (Pollock *et al.*, 1983).

In perennial grass species vernalization during the winter has been shown to increase the LER in spring as the plants enter the reproductive phase (Peacock, 1975; Peacock, 1976; Thomas and Norris, 1977; Parson and Robson, 1980). Controlled environment studies have shown that in such species chilling treatments can result in enhanced leaf extension without reproductive development when the day length is held constant (Stapleton and Jones, 1987). However, in this vernalization response the LER is enhanced at any given temperature, and there is no suggestion that the thermal response of leaf extension has adjusted to prevailing conditions. In fact a comparison of the thermal response of leaf extension in spring and autumn shows a lack of acclimation. Under field conditions Tomas and Norris (1977) found that LER of forage grasses was closely correlated with soil temperature throughout the year, but that LER was higher at any given temperature in spring compared to autumn. The lack of any adjustment in the thermal response to suit the

prevailing cold conditions in autumn is evidence against acclimation under field conditions.

As discussed in Chapter 1, *Lolium temulentum* plants transferred to 5 °C do not show significant adjustment of LER over 20 days Those transferred to 2 °C appear to adjust LER upwards over the first 3 days but the data is not detailed enough to draw a firm conclusion (Thomas and Stoddart, 1984). Seemingly more conclusive is the observation that *L. temulentum* leaves emerging at low temperature do not show improved LER compared with those transferred after leaves have emerged (Thomas, 1983). Thomas and Stoddart (1995) compared the LER in two *Festuca arundinacea* ecotypes following the transfer of plants to 5 °C for four days. Although the authors did not assess acclimation the data presented shows no evidence of LER increasing in either the cold adapted Scandinavian ecotype or the Mediterranean ecotype.

Despite the failure to directly observe an upward shift in LER during long-term cooling, there are several cases where LER measured during prolonged cooling is higher than during short-term cooling. This was illustrated for *L. temulentum* in Table 1.1, and for *Poa alpina* in Figure 1.2. Pollock and Eagles (1988), show that *L. temulentum* exhibits a sligtly lower T_b when grown at low temperature, suggesting some acclimation has occurred. Clifton-Brown, (1997) makes a more direct assessment of acclimation by comparing the short-term thermal response of the same *Miscanthus* genotype with or without acclimation (Figure 5.2). In this case the plants grown at 14 °C show a clear increase in LER at 14 °C, when compared to the warm grown plants.

In Figure 5.2 the cold acclimated plants also show enhanced LER at higher temperatures. This enhanced leaf extension following a period of growth at suboptimal temperatures has been noted in several studies (Kleinendorst, 1975; Thomas and Potter, 1985; Stoddart *et al.*, 1986; Thomas and Stoddart, 1995; Clifton-Brown, 1997). A similar response is seen in photosynthetic capacity with chilled plants showing elevated rates when returned to optimum temperatures (Pollock, 1983). Such phenomenon are probably distinct from the acclimation that takes place during prolonged cooling, as they are seen to occur after only short periods (< 1h) at low temperature and diminish within a few hours (Kleinendorst, 1975; Stoddart *et al.*, 1986; Thomas and Stoddart, 1995; Clifton-Brown, 1997). More likely the accelerated extension relates to the rewarming process itself, or perhaps some

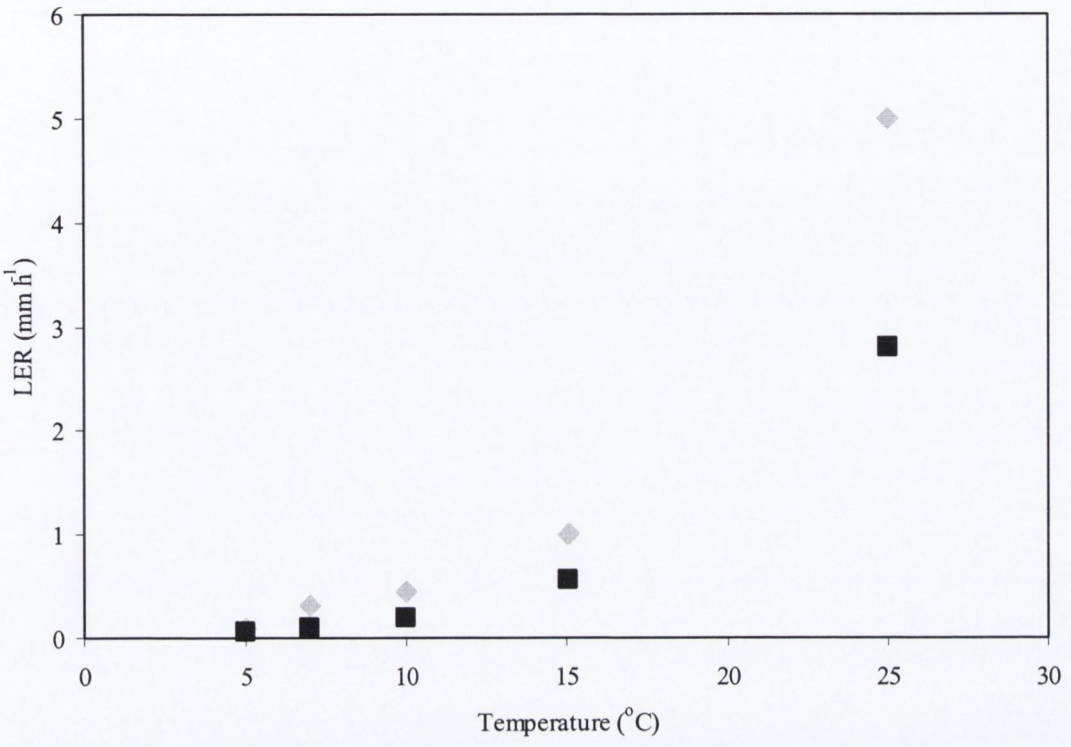


Figure 5.2 Leaf extension rate of *Miscanthus x giganteus*, grown at 25 °C (black squares) and at 14 °C (grey diamonds), when temperature was stepped from 5-20 °C over 2 h. Re-plotted from Clifton-Brown (1997).

element of 'stored growth' that is released once temperature is increased (see, Kleinendorst, 1975; Pollock and Eagles, 1988; Clifton-Brown, 1997).

Clearly the inverse correlation between the rate of leaf extension at low temperatures and the development of frost tolerance is linked with acclimation. Studies investigating the development of frost tolerance during acclimation have uncovered complex changes in the activity of several hormones, including several hormones known to be involved with modulating LERs (Levitt, 1980).

In particular, there appears to be an interaction between gibberellin and abscisic acid in determining the degree to which frost tolerance is developed during chilling. Furthermore, application of gibberellin has been shown to prevent the slowing of growth often associated with the development of frost tolerance (Levitt, 1980). Perhaps this same process plays a role in determining the extent to which LER is inhibited or enhanced by cooling over time. As mentioned in section 5.2, processes regulated by hormone levels may be less sensitive to temperature than processes that are regulated directly by biochemical reactions. This may allow for a degree of flexibility in the way different genotypes respond to long-term cooling.

Overall it seems clear that some adjustment of LER occurs during chilling but the full kinetics of the process remains to be determined. The physiological mechanisms involved in the acclimation process are discussed further in section 5.5. Given the lack of a clear picture it would be useful for future studies to monitor the change in LER during prolonged cooling, perhaps comparing the response of northern and southern, or upland and lowland genotypes.

5.4 Temperature and Turgor pressure

This section discusses the role of turgor pressure in determining LER during cooling. In particular examining the possibility that turgor may become limiting when the root system is cooled below the temperature of the expanding leaf or when evaporative demand is high.

As discussed in Chapter 3 there is now ample evidence that turgor pressure does not drop during long or short-term cooling of grass leaves. The same appears to be true for the expanding cells of hypocotyls (Cosgrove and Cleland, 1983; Boyer, 1993). Proseus *et al.* (2000), did detect a small drop in turgor pressure during the cooling of single internode cells of *Chara corallina*, however, turgor pressure is not thought to have caused the drop in expansion rate (see section 5.5.2) and when the turgor pressure was artificially returned to the control level expansion was not restored.

5.4.1 Leaf extension and root cooling

The root cooling experiment carried out by Pardossi *et al.* (1994) raises the possibility that turgor may limit leaf extension when soil temperatures are below that of the meristem. Pardossi *et al.* (1994) cooled the roots of *Phaseolus vulgaris*, while measuring LER and turgor pressure in expanding leaf cells (pressure probe). They found LER decreased with root temperature and at 10 °C was near zero. The turgor of the expanding cells also fell with root temperature and was much reduced at 10 °C. They observed that root cooling quickly reduced root water absorption while stomatal conductance and transpiration were slower to react. This caused the plant to dehydrate and lose turgor within 40 min of the roots being cooled. Turgor pressure largely recovered once stomatal closer had taken effect, which in turn led to an increase in LER. However, LER was still about half that of control levels. It is unclear whether this was due to the still lower turgor pressure or whether other changes had occurred due to the limitation in water supply, e.g. changes in wall properties as occurs in other species during drought (Matthews *et al.*, 1984; Thomas *et al.*, 1999) and salt stress (Cramer and Bowman, 1991).

Studies with grasses have also found that cooling the root system alone causes an immediate reduction in LER (Kleinendorst and Bouwer, 1970; Watts, 1972a; Clifton-Brown, 1997). Presumably this inhibition is also due in part to a drop in turgor pressure. Watts (1972b) however, points out the difficult with root cooling experiments in that the flow of cool water from the root and conductance through the tissue mean that the meristem itself will be subject to significant cooling.

In the summer, daytime soil temperatures will be lower than at the soil surface (Etherington, 1982). For a climate such as that in the UK it would not be surprising for deeper roots to be 10 °C colder than the leaf extension zone (LEZ) (Peacock, 1975a). This

suggest that the root cooling alone could result in LER inhibition above that imposed by meristem temperature. Peacock (1975b) tested this directly for a *Lolium perenne* crop by heating the soil surface, and found that the effect of root cooling was less severe that that of meristem cooling and so was not limiting for leaf extension.

The possibility remains that some plants (e.g. *P. vulgaris*, Pardossi *et al.* (1994)) may be more sensitive to root cooling and under certain conditions will be limited by water uptake during cold periods at least until the stomata response to the imposed water stress.

5.4.2 Root cooling and evaporative demand

The reduced water supply imposed by chilling the roots may be more crucial in determining LER when evaporative demand is high. In all of the studies discussed in Chapter 3 the inhibition of leaf extension occurs in the lab under conditions where light and VPD are generally low. In the field under high evaporative demand low root conductance and water absorption could produce a turgor driven limitation on LER above that of cooling the LEZ itself.

Although Watt (1972b) concluded that water stress is not the main factor limiting LER during root or shoot cooling he did identify two instances were evaporative demand had an important interaction with temperature in determining LER. In both cases air temperature was high and was in excess of root temperature while humidity was low. This caused evaporative demand to exceed water uptake so reducing turgor pressure in the LEZ. Such condition could occur in the field on cold dry mornings and also under experimental conditions where light and humidity is manipulated independently to temperature.

Ben-Haj-Salah and Tardieu (1996) carried out a series of field and growth chamber studies with maize plants, which have helped to clarify the interrelationship between temperature and evaporative demand in determining LER. The effect of light and VPD are considered by measuring LER at a given evaporative demand and comparing it with the maximum LER achieved with a low evaporative demand at the same meristem temperature (LER_{max}). They confirmed that when evaporative demand was low, LER was linked only to meristem temperatures regardless of other climatic conditions. When stomata were open, high VPD did reduce LER. The experiments led to the novel

conclusion that at any given temperature $LER_{measured}$ - LER_{max} is linearly correlated with VPD, i.e. if the effect of VPD is isolated from that of temperature there is a linear relationship between LER and VPD.

They found no change in the relationship in the lower temperature range (down to 10 °C) suggesting that chilling effects on water uptake did not become a limiting factor. They also found no direct effect of light from 0-1500 μmol m^2s^{-1} on LER over 2h, in growth cabinets or in the field, provided the effects on meristem temperature and evaporative demand was accounted for.

Given the above discussion, it is important for low temperature studies to consider the impact of evaporative demand (Thomas and Stoddart, 1984; Thomas and Stoddart, 1995; Clifton-Brown, 1997: Clifton-Brown and Jones, 1999) and the difference between root and LEZ temperature (Watts 1972a,b; Clifton-Brown, 1997; Kleinenedorst and Brouwer, 1970). In most cases the effect of temperature and VPD on LER will be independent of each other, with temperature acting only on the cell wall. However, in some plants low root temperature combined with high evaporative demand may cause an additional turgor driven inhibition of leaf extension.

5.5 Temperature and the cell wall

This section discusses the current state of knowledge regarding the effect of long and short-term cooling on wall properties. In particular it focuses on the relationship between wall mechanical properties and wall extensibility and aims to explain why cooling produces changes in wall mechanical properties in some but not all cases.

5.5.1 Cell wall extensibility measurement and meaning

The realisation that cell walls can change ϕ without any change in their mechanical properties, is central to understanding the response of cell expansion at suboptimal temperatures. In this study a basic distinction was made between purely mechanical wall properties (measured here as creep in boiled tissue and as mechanical deformation under applied force), and wall properties that include a metabolic component (measured here as

long-term creep in freeze/thawed tissue and as prolonged linear extension under applied force).

A distinction must also be made between wall loosening and wall extension. In this respect it is useful to remember the distinguishing feature of a Wall Loosening Factor (WLF), i.e. many components of the wall will influence the rate of cell expansion under certain conditions, but a true WLF must be able to initiate the reorganisation of the wall polymers rather than just constrain the extent of the extension once the wall loosening has begun. To date only expansins have been shown to perform this function (Cosgrove, 1997, Cosgrove, 2002). Crucially because WLFs initiate expansion a drop in their activity can reduce the rate of wall loosening without altering the physical architecture of the wall.

Although this definition emphasises the crucial role of WLFs it also makes it clear that other components in the wall can play a role in determining the cell expansion rate. Alterations in other wall components may, for example, modulate the amount of extension occurring during each loosening event or the frequency at which loosening events occur. Such alterations may involve significant modification of the bonding arrangements in the wall in which case they can be detected using mechanical assays (Cosgrove, 1993a).

This interpretation differs from that of Cleland (1984) who suggests that Instron plasticity measures those domains in the wall where a wall-loosening event has occurred but has not been eliminated by further biochemical events. In this case any change in the activity of the WLF would in time be reflected in a change in Instron plasticity. There are however several cases where tissue has shown a large change in cell expansion under constant turgor with no change in Instron plasticity (Cosgrove, 1988; Arif, 1990; Pritchard *et al.*, 1990a; Thomas, 1990). These studies rule out the possibility of any fixed relationship between the rate of the wall loosening and the degree of Instron plasticity such as that suggested by Cleland (1984). Instead the results point towards dual control of leaf extension as WLFs function within the constraints of the wall structure (see Chapter 3, section 3.4). Having said this, where correlation does occur between ϕ and mechanical extensibility it seems reasonable to suggest that the differences in bonding arrangement that result in increased mechanical extensibility are also responsible for the increase in extension rate.

In addition to WLFs and significant structural changes, cell expansion rate can be influenced by more subtle differences in wall composition and by ephemeral wall properties such as wall hydration (Chazen and Neumann, 1994; Passioura, 1994), porosity of the wall matrix (Pritchard, 1994; Caprita and McCann, 2000), or the concentration of H^+ and other inorganic ions (Pritchard, 1994; Wu and Cosgrove, 2000). Such changes are not measured directly by either method in Chapter 3 but may play a role in the responses seen in the *in vivo* assay used in Chapter 4.

In this study differences in mechanical properties are taken only as an indication of differences in wall structure. Extensibility of active tissue however has shown good correlation with LER in this and other studies (Matthews *et al.*, 1984; Cosgrove, 1989; Pritchard *et al.*, 1990a; Cosgrove and Li, 1993; Cho and Kende, 1997a,b) and is taken as a true measure of ϕ in equation 1.2. The parameter ϕ can be influenced by changes in any of the three components outlined above: (1) The action of WLFs; (2) Non-structural properties which modulate the action of WLFs; and (3) Structural properties which modulate the action of WLFs or the extension which follows.

5.5.2 Inhibition of wall expansion during short-term cooling

Perhaps the earliest investigation into the effect of temperature on wall properties were carried you in Robert Cleland's laboratory in the late 1960s (see Cleland, 1971a; Cleland, 1977). Cleland (1971b) reports on a series of traditional creep experiments measuring the viscoelastic properties of *Avena* coleoptiles. He found that a change in the temperature at which the assay was carried out caused only a slight increase in the amount of extension with a Q_{10} of about 1.05 between 4 °C and 24 °C. Stress relaxation tests with walls of *Nitella* also found that temperature changes over this range had little effect on the mechanical properties of the tissue (Haughton *et al.*, 1968; Cleland, 1971a). Much later Thomas (1990) compared the mechanical compliance of killed tissue from *L. temulentum* seedlings, following 30 minutes treatment at 5 °C. He found no difference in the Instron plasticity of walls treated with 5 °C compared with 20 °C, even when the tissue was measured at 20 °C and 5 °C. Similarly, Pritchard *et al.* (1990a) found that cooling tissue form the root extension zone of maize to 5 °C for 50 minutes resulted in a marked fall in

extension rate but no change in Instron plasticity, even when the tensiometric measurements were carried out at 5 °C.

In all of these cases the tissue was pretreated in ways that remove protein activity from the wall. Under these conditions it is clear that the mechanical properties of the cell walls are not sensitive to short-term changes in temperature and are not responsible for the initial inhibition of cell expansion seen during cooling.

The experiments described in Chapters 3 and 4 differ from those discussed above in that the tissue used retained active wall proteins including those involved in loosening the wall. Using this approach there is a clear correlation between the thermal response of extensibility and the thermal response of leaf extension itself. No such correlation was found for the thermal response of wall mechanical properties (Figure 3.8 and Figure 4.10). The two conclusions together can be taken as evidence that LER during short-term cooling is limited by ϕ, and that the limitation does not involve significant changes in wall structure. The mere fact that wall extension can be altered so rapidly by cooling and other metabolic inhibitors tends to support a model of cell expansion limited by chemical reactions, such as the breakage of bonds, rather than a metabolically regulated viscoelastic flow which results from overall properties of the wall (Ray and Ruesink, 1962). Under the current model of wall loosening these results suggest that the inhibition of ϕ is mediated by a change in the activity of WLFs. Most likely through an increase in the activation energy needed for proteins such as expansins to disrupt load bearing bonds in the wall.

This hypothesis is supported by the *in vivo* relaxation experiments carried out by Cosgrove (1987). In these experiments the growing tissue was excised to prevent water uptake, wall loosening continues, causing cell turgor pressure to fall until the yield threshold is reached. The faster the turgor pressure falls the greater the extensibility of the wall (Cosgrove, 1993a). During trials with this technique Cosgrove (1987) compared the fall in turgor pressure in soybean hypocotyls at 28 °C and 8 °C, and found the rate of reduction of the cooled tissue was greatly reduced. That is ϕ was reduced by cooling. Although this reduction could be due to structural changes in the wall, the fact that in the reduction was seen in Cosgrove's experiments with metabolically active tissue and not in the killed tissue tested in Chapters 3 or 4, or in other studies (Thomas, 1990; Pritchard *et*

al., 1990a, Cleland; 1971), supports the conclusions that it is the activity of WLFs which is effected.

In large internode cells from *Chara corallina*, Proseus *et al.* (2000), were able to alter turgor pressure artificially by injecting additional cell sap into a growing cell thus causing cell osmotic potential and turgor pressure to rise. As with the applied force method in Chapter 4 they found growth rate increased under the additional force (in this case the response was curvilinear). By applying step increases in turgor pressure they were able to estimate the *in vivo* wall extensibility. As with the barley leaves in Figure 4.3, when the alga cell was cooling there was a marked drop in *in vivo* extensibility.

Perhaps the strongest support for the conclusions, outlined above, comes from the set of measurements made by Pritchard *et al.* (1990a) during short-term cooling of maize roots. As outlined above no change was seen in Instron plasticity even after 50 minutes. However, a second measure of ϕ was also carried out: here the root tissue was incubated in various concentrations of mannitol to artificially alter the turgor pressure and cell extension rate. A plot of extension rate against turgor pressure produced a linear relationship the slope of which represents ϕ. Again in this assay the wall remains biochemically active and again Pritchard *et al.* (1990a) found a marked fall in ϕ during short-term cooling.

It is interesting to contrast the cooling response with that seen during the inhibition of extension growth in oat coleoptiles when transfer from dark to light (Cosgrove and Li, 1993). In this case the reduced extension rate was not associated with decreased activity of WLFs; as expansins extracted from dark and light treated coleoptiles had the same effect during creep assays (applied to heat inactivated wall tissue from cucumber). Instead the ability of the tissue to respond to expansins was reduced in the light treated coleoptiles; as measured using a creep assay (using heat-inactivated oat coleoptiles with equal amounts of expansin applied). In this case it is clear that a change in some factor other than the activity of WLFs causes the inhibition of growth. Exposure to light may have resulted in increased cross-linking in the wall limiting extension steps, or reduced wall porosity limiting the access of expansins to load bearing bonds (Cosgrove and Li, 1993). A subsequent study showed that growth inhibition following transfer to light was associated with changes in the mechanical properties of the walls (in maize coleoptiles)

(Parvez *et al.*, 1996). The changes in the susceptibility of the oat walls took approximately 8 h to develop. During the 8 h when the coleoptiles were exposed to light but few changes in wall properties had occurred the extension rate can be seen to gradually fall reaching a minimum at about 8 h (Cosgrove and Li, 1993). It appears that that structure of the wall is gradually altered resulting in a significantly less plastic wall after 8 h. This contrasts with the response seen during short-term cooling, where the inhibition of extension growth was instant and no change in mechanical properties were detected (Chapter 3 and 4).

5.5.3 Inhibition of wall expansion during long-term chilling

Identifying the mechanism that results in the initial reduction of LER during cooling was a central aim of this study, however, the results also have some relevance in interpreting the responses seen during long-term chilling. When cells are chilled for prolonged periods we must consider the role of the three components of ϕ and the degree of acclimation in each. The understanding that mechanical properties will correlate with LER in some but not all cases is a key consideration when comparing studies using long (>7 d) and short-term cooling (<2h).

Pritchard *et al.* (1990a), carried out long-term experiments with maize roots chilled over 10 days. In this case the Instron plasticity began to fall after about 24 h and continued to decline gradually over the next 4 days, by which time it was approximately 10% of the original value. When the roots were returned to 20 °C, Instron plasticity remained low for 2 d after which it began to rise gradually, recovering fully after 4 days. The recovery of cell expansion followed a similar trajectory. Clearly chilling for prolonged periods can affect wall structure. Also, given the correlation between Instron plasticity and cell expansion during the recovery phase, these changes in wall structure are probably involved in modulating ϕ. However, it is important to keep in mind that while the cells are at 5 °C ϕ will also be restricted by the direct effects of temperature on wall loosening as seen in Chapter 3 and 4. In Pritchard *et al.*'s (1990a) experiments the changes in wall structure do not appear to result in any recovery in cell expansion, which remained constant over the 10 days. An analysis of cell lengths during the recovery phase suggests that the cells produced during the 10 d cold period do not resume expansion when returned to 20 °C. Clearly in the case of maize roots changes in Instron plasticity cannot

be viewed as acclimation to low temperature and are perhaps better considered as a secondary restriction on growth brought about by a prolonged stress.

This role for Instron plasticity contrasts with that proposed by Woodward and Friend (1988). In their long-term cooling experiment (10 d) with *Poa* plants from three different altitudes. They showed that the ability of the high altitude plants to maintain leaf growth below 6 °C, is associated with an ability to retain their original Instron plasticity. Woodward and Friend (1988), take this as an indication that Instron plasticity of the expanding cell walls may limit leaf extension at low temperatures. This conclusion is not supported by the results of Chapter 3 and 4 or the other short-term studies discussed above. The changes in Instron plasticity during cooling cannot be taken as changes ϕ and should only be taken as an indication of an alteration in wall structure. In support of this argument, it is interesting to note that although Instron plasticity in the high altitude *Poa* was identical at 20 °C and 5 °C leaf extension was reduced by 40% (much less than the non-adapted species but not inconsiderable).

Rajashekar and Lafta (1996) found that suspension-cultured cells from grape and apple subject to chilling at 2 °C for 6 weeks had an increase cell wall breaking pressure compared with cells kept at 20 °C. In this case increased rigidity of the wall during chilling was interpreted as being a frost resistance mechanism increasing the resistance of the cells to intracellular freezing and to collapse during extracellular freezing. Several studies have detected changes in the composition of cell walls during chilling (Zabotin *et al.*, 1989; Weiser *et al.*, 1990; Nakamura *et al.*, 2003). These include quantitative and qualitative change in cross-linking glycans (Zabotin *et al.*, 1989; Nakamura *et al.*, 2003) and an increase proportion of the structural protein, extensin (Weiser *et al.*, 1990), both of which would be expected to increase the rigidity of the wall.

No such changes in mechanical properties were detected in the barley seedlings tested in Chapter 4. During 7 h at 10 °C the mechanical deformation was seen to be unchanged. This might be expected given the less sever temperature treatment used and the generally low temperature sensitivity in barley.

Taken together the changes in wall composition and wall strength (Instron plasticity and breaking point pressure) certainly show that wall structure is altered during prolonged

chilling. None of the studies suggest that the alterations in the wall will increase cell expansion after a period at low temperature, as might be expected in a typical acclimation response. Rather the changes show a stiffening of the wall, which may increase tolerance to frost but may also reduce ϕ and restrict cell expansion rate.

This interpretation appears to correspond with the negative correlation between growth rate at suboptimal temperatures and subsequent frost tolerance discussed in section 5.3.2. The degree to which different plants are subject to this secondary restriction will vary as it is not regulated directly by temperature but seems to involve secondary responses in the growing cells and perhaps long distance hormonal signalling. It may be that the degree to which gibberellin is reduced (or abscisic acid increased) during chilling influences the extent to which the wall is altered and thus the extent of the long-term inhibition (as discussed in section 5.3.4).

A recent study by Nakamura *et al.* (2002), came to similar conclusions to Woodward and Friend (1988), namely that temperature modulates the growth rate of rice coleoptiles by affecting mainly the mechanical properties of the cell wall. Nakamura's principle finding was that Instron plasticity fell with temperature following chilling for 8 h at 10 °C. This differs somewhat from the maize roots studied by Pritchard *et al.* (1990a) where changes were not detected until about 24 h. An even more marked difference was seen during recovery. When Nakamura *et al.* (2002) transferred cold treated plants back to the original temperature, they detected a full recovery in Instron plasticity after 4 h, while Pritchard *et al.* (1990a) found samples took 4 days to fully recover and showed little change in the first 48h. A similar difference was seen in the length of time taken for the growth rate of the tissue to recover in the two studies indicating that the variation in time is due to species differences.

The timescale of the Nakamura *et al.* (2002) study is neither long nor short-term in the limits defined here. It is perhaps surprising that changes in wall structure were detected after 8 h cooling and after just 4 h of rewarming, but it is not outside the expected range for wall assembly (Cosgrove, 1997). Increases in cell expansion rate following application of auxin or fusicoccin were accompanied by increase in the rate of polysaccharide biosynthesis after 30 –120 minutes (Brummell and Hall, 1983; Eldemann *et al.*, 1989). If we take this as the minimum time for the modification of wall

composition, it is not surprising that Nakamura *et al.* (2002) saw structural changes after 4 h. Nakamura *et al.* (2002) did not test the Instron plasticity during the 4 h recover period, but understandably assumes the recovery in growth follows from the recovery in Instron Plasticity. This is a crucial point because if the cell expansion recovers before Instron Plasticity the implication is that expansion was restricted by the activity of WLFs alone, whereas if the Instron plasticity recovers inline with cell expansion the suggestion is that this structural component of the wall properties comes to limit cell expansion during chilling.

It may be that although Instron plasticity is not the source of limitation for cell expansion during short-term cooling, the changes in wall structure indicated by the Instron measurements do play a role in determining the response of LER under prolonged chilling. Indeed it is tempting to suggest that the high altitude *Poa* species has adapted to low temperature in such a way that it maintains an extensible wall architecture under prolonged cooling (Woodward and Friend, 1988) while the temperature sensitive species response by stiffening the wall as seen in the maize and rice (Pritchard, 1990a; Nakamura *et al.*, 2002). However, none of the studies directly correlate the changes in the wall with a reduction in cell expansion rate beyond that which occurs immediately following cooling. This is despite the fact some of the changes are reported to develop over several hours (Pritchard *et al.*, 1990a; Zabotin *et al.*, 1998) or days (Weiser, 1990).

It would be interesting to investigate whether the maintenance of Instron plasticity in the high altitude *Poa* species (Woodward and Friend, 1988) is associated with a recovery of LER, or perhaps more likely if the fall in Instron plasticity in the temperature sensitive species causes a further reduction in LER, over the 10 day period.

Taken together the results of Pritchard *et al.* (1990a), Woodward and Friend, (1988) and Nakamura *et al.* (2002), show that changes in wall structure that occur during chilling can restrict LER during rewarming. These changes may also have a role in regulating the LER during prolonged exposure to suboptimal temperatures.

5.6 Genotypic variation in thermal responses of wall extensibility

There was a clear difference in the thermal response of LER between maize and barley. The lower temperature sensitivity in barley was reflected in the results of the applied force experiments, with maize showing a larger relative drop in *in vivo* extensibility (Q_{10}= 3.9; between 14-21 °C) than in barley (Q_{10}= 2.8; 10-20 °C). In this section the source of such genotypic differences is investigated. A model is suggested incorporating the conclusion from the preceding sections; that wall extensibility is a result of the interaction between wall loosening activity and structural arrangements in the wall.

5.6.1 The example of *slender* barley

Within the grasses that have been tested *slender* barley appears to be unique in its ability to continue leaf extension at temperatures as low as –5 °C (Stoddart and Lloyd, 1986). This provides an ideal system with which to analyse genotypic differences in the thermal response of leaf extension. A comparison between the wildtype and *slender* barley is outlined below and possible mechanisms through which differences in thermal response may be modulated are proposed.

In the time since Stoddart and Lloyd (1986) described the reduced temperature sensitivity in *slender* genotypes the nature of the mutation has been better characterised. It is now know that the *slender* phenotype results from a loss-of–function mutation in the functional domain of a DELLA protein (Lananhan and Ho, 1988; Fu *et al.*, 2002; Chandler *et al.*, 2002). DELLA proteins are thought to act as transcription factors. Interestingly, rather than being stimulated by gibberellin, the DELLA proteins function to repress leaf extension in the absence of gibberellin. When gibberellin is present the DELLA proteins are converted to the inactive form releasing the constraint on LER (Richards *et al.*, 2001; Sun and Gubler, 2002). The *slender* mutation inactivates the DELLA protein thus simulating the effect of applying saturated gibberellin; causing LER to continue unchecked.

Given that the slender mutation affects both the maximum LER and the thermal response of LER, it seems likely that the control (repression) of LER at ambient temperatures is interlinked with the control of LER during cooling. The most straightforward explanation for this would be that the temperature sensitivity of gibberellin production or transport is

the limiting step for leaf extension at suboptimal temperatures, with the reduced availability of gibberellin resulting in an increase in the repression function of the DELLA proteins. In the *slender* genotype where no gibberellin is required the LER remains un-repressed. The observation that gibberellin application alters the thermal response of amylase secretion in barley aleurone layers (Fadeell *et al.*, 1980) lends support to a general interaction between gibberellin supply and temperature sensitivity. If this were the case wildtype barley plants growing at 10 °C would have a lower concentration of bioactive gibberellin in the LEZ relative to that seen at optimum temperatures. However, this does not appear to be the case. Tonkinson *et al.* (1997), tested the endogenous gibberellin content in the LEZ of wheat grown at 20 °C and 10 °C and found no significant difference. Tonkinson *et al.* (1997) advances the alternative hypothesis that low temperature increases the sensitivity threshold for gibberellin action. Given that the temperature sensitivity is essentially absent in the *slender* barley mutant this would have to occur upstream from the DELLA proteins.

The hypothesis that gibberellin supply or sensitivity is involved in regulating leaf extension during cooling seems worthy of further investigation especially with regard to long-term chilling. However, it is difficult to see how a temperature sensitive step in the gibberellin transudation pathway could result in an alteration in the short-term thermal response. *Slender* barley exhibits its novel thermal response in leaf extension without any acclimation period (Stoddart and Lloyd, 1986). The responses during short-term cooling are too rapid for changes upstream of the DELLA transcription factors to act on ϕ. An alternative explanation is that changes in the *slender* plants that result in rapid LER at ambient temperature also act to modify the thermal sensitivity of the cell wall itself.

How does this fit with the conclusions outlined above that the short-term thermal response is determined by the direct effect of temperature on basic biochemical processes i.e. the breaking of bonds by a WLF? It is surprising that such a fundamental thermal restriction could be so profoundly effected by a change in an endogenously regulated signalling pathway. It appears that by removing the endogenous restrictions on LER the *slender* mutation has altered the activation energy of the WLFs themselves. This phenomenon may be crucial to understanding the variation in thermal response seen between other genotypes, several possible explanations are outlined below.

(1) It is possible that removing the influence of the DELLA proteins as transcription factors causes the conformation of WLFs to be altered in a way that reduces their activation energy. This would seem unlike given that the levels of gibberellin and in turn of DELLA protein activity is naturally variable between plants depending on genotype and growth conditions, yet no such conformational change has been described. Perhaps more plausible would be a change in the composition of various WLFs, e.g. a change in the relative abundance of certain members of the expansin family which had a lower activation energy. Our limited knowledge of the functional role of each expansin protein (Cosgrove, 1999) does not allow an assessment as to the likelihood of regulation through this kind of mechanism.

(2) Gibberellin may modify the wall environment by causing non-structural changes in the wall environment that influence the functioning of WLFs. The most obvious candidate is wall pH, however studies have shown that gibberellin stimulated extension does not occur through wall acidification (as is the case for auxin) (Davis, 2002). An interaction between the fluidity of the membrane or wall matrix and control of cell extensibility during cooling has already been discussed (section 5.3.1). The possibility that gibberellins influence extension by altering this or other subtle aspects of wall composition cannot be ruled out.

(3) The control of LER by the DELLA protein may be via alterations in the structure of the wall rather than in the action of WLFs. The bonding arrangements in the wall are constantly re-established as each new layer of wall is synthesised. A change in wall composition or bonding arrangements may increase the LER at optimal temperatures, while also altering the activation energy needed to break the bonds, so altering the thermal response. This possibility was partially tested in Chapter 4 and some differences in the bonding arrangements were suggested: in Figure 4.11 and in the results of the Instron analysis carried out by Pollock *et al.* (1990). In addition, Behringer *et al.* (1990) found that gibberellin stimulated cell elongation was associated with an increase in *in vivo* extensibility (stress relaxation using pressure block method) and in mechanical properties (short-term creep test on freeze/thawed tissue). This provides evidence that gibberellin alters wall structure as well as the thermal response. However, the effects of gibberellin

on LEZ length prevent a conclusive interpretation from any of these experiments (see below).

A separate line of evidence indicating that gibberellin significantly alters wall structure is the correlations between gibberellin stimulated cell expansion and XET expression. *Slender* barley seedlings and dwarf seedlings treated with gibberellin show increased expression of XET in the base of the LEZ compared with untreated dwarfs (Smith *et al.*, 1996; Schunmann *et al.*, 1997). Although XET was originally investigated as a possible WLF, more recent studies point to a role in wall assembly. Most recently it has been suggested that XET may function as a 'secondary wall loosening agent' acting to modify the wall architecture in a way that enhances the action of the primary WLF (Cosgrove, 1999; Wu and Cosgrove, 2000). In this way changes in the bonding arrangements brought about by the increase in activity of XET may produce a LEZ that is; (A) more extensible at optimum temperatures; and (B) less sensitive to temperature change.

These three proposals serve to emphasise the array of possible mechanisms through which ϕ at low temperatures might be modulated. They include the three possible points of control in the wall: (1) action of WLFs; (2) non-structural changes which alter the action of WLFs; and (3) structural changes which alter the effectiveness of WLFs or the extension which follows. The third proposition is particularly intriguing given the strong evidence for an effect of gibberellin on XET at ambient temperatures. In order to further explore this possibility we must first analyse the behaviour of the LEZ as a whole.

5.6.2 The importance of leaf extension zone length

As discussed in Chapter 4 although tissue from *slender* barley does exhibit an altered wall structure compared to wild type, the alteration appears to affect the distance over which cells expand rather than the speed of extension during the maximum growth phase. The changes in wall structure are still significant and may still be linked to the modification in the thermal response. However, it is important to consider this variation in LEZ length when comparing the wall properties. The measurements of mechanical properties made by Pollock *et al.* (1990) comparing *slender* and wildtype barley, and by Behringer *et al.* (1990) comparing gibberellin treated and control plants, failed to take account of probable

differences in the profile of cell expansion between the plants tested. In Chapter 4, we saw the significance of LEZ length in interpreting the relationship between *in vivo* extensibility and LER. With the longer extension zone in *slender* resulting in a lower average *in vivo* extensibility across the tissue and thus a lower mechanical compliance relative to tissue length.

Pritchard (1994) emphasised the importance of considering the response of cells in different parts of the LEZ; as several treatments have been shown to have contrasting effects in cells of different ages. It may be that cells at different stages of their growth cycle have inherently lower temperature sensitivity. For example, cells in the young accelerating phase may require less energy to loosen load bearing bonds; essentially reducing the base temperature at which extension can take place. Differences in the structural properties of wall tissue from the *slender* mutant could result from an increased number of such cells.

In addition to the above considerations, it has been suggested that change in LEZ length during prolonged chilling may play a part in determining the leaf extension rate response (Clifton-Brown, 1997). LEZ length has been shown to change in response to various treatments such as water stress (Durand *et al.*, 1995), light intensity (Schnyder and Nelson, 1988), and CO_2 levels (Ferris *et al.*, 1996). The possibility of differences in LEZ length, and so in the profile of cell expansion, was not considered by Woodward and Friend (1989) when they measured the Instron plasticity of tissue grown at 20 °C and 10 °C. Differences in length may account for some of the differences in Instron Plasticity which where reported in this study. Similarly, Nacamura *et al.* (2003) fail to consider differences in the profile of the cells being compared between different temperature treatments, this is particularly crucial in this case as the coleoptiles are young and the effect of growth at low temperature for 8 h is a 20 mm shorter coleoptile. Clearly this difference in developmental age may influence the extension properties of the tissue being tested regardless of temperature.

Clifton-Brown (1997) tested this theory in *Miscanthus* and found no change in LEZ during cooling. Tonkinson *et al.* (1997) did detect a shortening of the LEZ in wheat grown at 10 °C. They also found that the length of the LEZ at 10 °C was restored by application of gibberellin. Given the interaction between gibberellin and thermal

responses discussed above, this suggests changes in the profile of cell expansion could have a role in determining temperature sensitivity during long-term cooling.

5.6.3 Thermal control of wall extensibility: a model

Throughout this discussion we have seen that temperature effects LER by altering the extensibility of cells in the LEZ. In the case of short-term cooling this occurs without changes in wall structure. In other cases differences in wall structure do contribute to differences in cell extensibility. In particular the correlation between the thermal responses of LER and the thermal responses of wall mechanical properties seen in the Alpine *Poa* species (Woodward and Friend, 1988) appears to support a central role for wall structure in modulating cell extensibility. Thus a model can be proposed:

- During acclimation to long-term chilling many plants will reduce the activity of secondary wall loosening agents, such as XET. This alters wall structure producing walls that are more ridged and less extensible. Such architectural changes were seen in the lowland *Poa* ecotype (Woodward and Friend, 1988), in rice (Nakamura *et al.*, 2002), in maize roots (Pritchard *et al.*, 1990) and in cell cultures (Rajasher and Lafta, 1996).

  The development of a more rigid wall may be related to the development of frost tolerance, thus explaining the negative correlation often found between LER at low temperatures and frost tolerance.

  Not all genotypes respond to chilling in this way, some maintain their wall extensibility in order to maximise leaf extension in the low temperature range (e.g. the upland *Poa* ecotype (Woodward and Friend, 1988)).

- During short-term cooling the sensitivity of LER in a given genotype will be equally depend on the past activity of secondary wall loosening agents. In *slender* barley XET activity is enhanced producing walls that are particularly insensitive to cooling (in the same way as the upland *Poa* ecotype is insensitive to long-term chilling).

In barley there is a suggestion that gibberellin is involved in regulating the activity of the secondary wall loosening agents, and again a link with frost tolerance seems likely (as increased gibberellin is thought to reduced frost tolerance, see section 5.3.4)

When comparing wall extensibility between genotypes the behaviour of the whole LEZ must be considered. It may be that rather than creating cells with reduced temperature sensitivity the secondary wall loosening agents act to increase the population of certain cells that are inherently less temperature sensitive (e.g. cells in the accelerating phase).

5.7 Final conclusion and future research

Wall extensibility at any given temperature is determined by both wall structure and the activity of WLFs. The short-term inhibition of cell expansion at suboptimal temperatures is not associated with a change in turgor pressure or in the mechanical properties of the cell wall but can be explained by the direct effects of temperature on wall loosening proteins. Long-term cooling does appear to involve changes in wall structure that are reflected in the mechanical properties. These changes may influence the extent to which LER is inhibited over time, but direct evidence of this is lacking.

Similarly, there is some evidence that genotypic differences in the short-term thermal response may be modulated by differences in wall structure, but shortcomings in the methods used make it difficult to draw firm conclusions.

Future studies aiming to identify the source of differences in thermal response between genotype or between treatment duration, must account for the interaction between WLFs and wall structure. This could be achieved using the methods from Chapter 3 or 4, provided a comparison is made between active and inactive tissue. The assay used by Cosgrove and Li (1993) provides a third method of comparing wall properties, essentially measuring the susceptibility of different walls to a controlled amount of wall loosening activity. This too may prove useful in understanding genotypic differences in thermal responses.

Future studies should also consider the behaviour of cells throughout the LEZ. Firstly because differences in the profile of cell expansion can affect the interpretation of the parameters measured, and secondly because there is evidence that the length of the LEZ is sensitive to temperature change. Given the difficultly in comparing the mechanical properties of tissue with different cell expansion profiles, an assessments of wall structure based on imaging techniques or biochemical and molecular assays may be more fruitful.

Several lines of evidence have uncovered an interaction between gibberellin activity and the nature of the short-term thermal response. Understanding the mechanism behind this interaction should provided useful information regarding the source of varying temperature sensitivity in general. In particular, there is growing evidence that gibberellin increases LER by altering the expression of XET along the LEZ. The possibility that increased XET expression is also responsible for the reduced temperature sensitivity seen in *slender* barley and in gibberellin treated cereals is worthy of further investigation.

In Addition to the central question regarding the source of variation in thermal response of LER, some basic questions regarding the response of LER to suboptimal temperatures remain unanswered. A detailed analysis of LER during long-term cooling would be invaluable in determining the extent to which changes in wall properties are associated with increasing or decreasing expansion rate. By measuring ecotypes with contrasting responses to cooling, as discussed in section 5.3., such an analysis may also uncover useful agronomic characteristics.

References

Abe, J. and Yoshida, M. (1997). Influence of water and air temperature on cold hardiness of wheat. In *Proceedings of the International Symposium on Cereal Adaptation to Low Temperature Stress in Controlled Environments* (ed. Z. Bedo, J. Sutka, O. Tischner and B. Köszegi), pp. 11-17. Martonvasar, Hungary: Agricultural research Institute of the Hungarian Academy of Sciences.

Angus, J. F., R. B. Cunningham, Moncur, M. W. and Mackenzie, D. H. (1981). Phasic development in field crops I. Thermal response in the seedling phase. *Field Crops Research* **3,** 365-378.

Arif, H. (1990). Water Relations of Salt Stressed Wheat: University of Wales Bangor.

Baker, N. R., Long, S. P. and Ort, D. R. (1988). Photosynthesis and temperature, with particular reference to effects on quantum yield. In *Plants and Temperature* (ed. S. P. Long and F. I. Woodward). Cambridge: Company of Biologists.

Barlow, E. W. R., Boersma, L. and Young, J. L. (1977). Photosynthesis, transpiration and leaf elongation in corn seedlings at suboptimal soil temperatures. *Agronomy Journal* **69,** 95-100.

Beale, C. V. and Long, S. P. (1995). Can perennial C4 grasses attain high efficiencies of radiant energy conversion in cool climates? *Plant, Cell and Environment* **18,** 641-50.

Behringer, F., Cosgrove, D., Reid, J. B. and Davies, P. J. (1990). Physical basis for altered stem elongation rates in internode length mutants of *Pisum*. *Plant Physiology* **94,** 166-173.

Ben-Haj-Salah, H. and Tardieu, F. (1996). Quantitative analysis of the combined effects of temperature evaporative demand and light on leaf elongation rate in well-watered field and laboratory-grown maize plants. *Journal of Experimental Botany* **47,** 1689-1698.

Berry, J. A. and Bjorkman, O. (1980). Photosynthetic response and adaptation to temperature. *Annual Review of Plant Physiology* **31,** 491-543.

Biscoe, P. and Gallagher, J. (1977). Weather, dry matter production and yield. In *Environment Effects on Crop Physiology* (ed. J. Landsberg and C. Cutter). London: Academic Press.

Boyer, J. S. (1993). Temperature and growth-induced water potential. *Plant, Cell and Environment* **16,** 1099-1106.

Bray, E. A., Bailey-Serres, J. and Weretilnyk, E. (2000). Responses to Abiotic Stresses. In *Biochemistry and Molecular Biology of Plants* (ed. B. B. Buchanan, W. Gruissem and R. J. Jones). Rickville, Maryland: American Society of Plant Physiologists.

Brett, C. T. and Waldron, K. W. (1996). Physiology and Biochemistry of Plant Cell Walls. London: Chapman and Hall.

Brummell, D. A. and Hall, J. (1983). Regulation of cell walls synthesis by auxin and fusicoccin in different tissues of pea stem segments. *Physiologia Plantarum* .

Bunce, J. A. (1977). Leaf elongation in relation to leaf water potential. *Journal of Experimental Botany* **28,** 156-161.

Cappelletto, P., Mongardini, F., Barberi, B., Sannibale, M., Brizzi, M. and Pignatelli, V. (2000). Papermaking pulps from the fibrous fraction of *Miscanthus x giganteus*. *Industrial Crops & Products* **11,** 205-210.

Carpita, N. and McCann, M. (2000). The Cell Wall. In *Biochemistry and Molecular Biology of Plants* (ed. B. B. Buchanan, W. Gruissem and R. J. Jones). Rickville, Maryland: American Society of Plant Physiologists.

Carpita, N. C. (1996). Structure and biogenesis of the cell walls of grasses. *Annual Review of Plant Physiology and Plant Molecular Biology* **47,** 445-476.

Cassab, G. I. (1988). Plant cell wall proteins. *Annual Review of Plant Physiology and Plant Molecular Biology* **49,** 281-309.

Chandler, P. M., Marion-Poll, A., Ellis, M. and Gubler, F. (2002). Mutants at the *Slender1* Locus of barley cv Himalaya. Molecular and physiological characterization. *Plant Physiology* **129,** 181-190.

Chazen, O. and Neumann, P. M. (1994). Hydraulic signals from the roots and rapid cell-wall hardening in growing maize (*Zea may* L.) leaves are primary responses to polyethylene glyco-induced water deficits. *Plant Physiology* **104,** 1385-1392.

Cho, H. and Kende, H. (1997a). Expansins in deepwater rice internodes. *Plant Physiology* **113,** 1137-1143.

Cho, H. and Kende, H. (1997b). Expansins and internodal growth of deepwater rice. *Plant Physiology* **113,** 1145-1151.

Christian, D. G. and Haase, E. (2001). Agronomy of *Miscanthus*. In *Miscanthus - for Energy and Fibre* (ed. M. B. Jones and M. Walsh). London: James and James (Science Publishers).

Clarkson, D. T., Earnshaw, M. J., White, P. J. and Cooper, H. D. (1988). Temperature dependent factors influencing nutrient uptake: an analysis of responses at different levels of organisation. In *Plants and Temperature* (ed. S. P. Long and F. I. Woodward). Cambridge: Company of Biologists.

Cleland, R. (1971a). Cell wall extension. *Annual Review of Plant Physiology* **22,** 197-222.

Cleland, R. (1971b). The mechanical behaviour of isolated *Avena* coleoptile walls subject to constant stress. Properties and relation to cell elongation. *Plant Physiology* **47,** 805-811.

Cleland, R. E. (1977). The control of cell enlargement. *SEB symposium XXXI* .

Cleland, R. E. (1984). The Instron technique as a measure of immediate-past wall extensibility. *Planta* **160,** 514-520.

Clifton-Brown, J. C. (1997). The importance of temperature in controlling leaf growth of *Miscanthus* in temperate climates: Trinity College Dublin.

Clifton-Brown, J. C. and Jones, M. B. (1997). The thermal response of leaf extension rate in genotypes of the C4-grass *Miscanthus*: an important factor in determining the potential productivity of different genotypes. *The Journal of Experimental Botany* **48,** 1573-1581.

Clifton-Brown, J. C. and Jones, M. B. (1999). Alteration of transpiration rate, by changing air vapour pressure deficit, influences leaf extension rate transiently in *Miscanthus*. *Journal of Experimental Botany* **50,** 1393-1401.

Clifton-Brown, J. C. and Lewandowski, I. (2000). Overwintering problems of newly established *Miscanthus* plantations can be overcome by identifying genotypes with improved rhizome cold tolerance. *New Phytologist* **148,** 287-294.

Clifton-Brown, J. C. and Lewandowski, I. (2002). Screening Miscanthus genotypes in field trials to optimise biomass yield and quality in Southern Germany. *European Journal of Agronomy* .

Clifton-Brown, J. C., Lewandowski, I., Andersson, B., Basch, G., Christian, D. G., Bonderup-Kjeldsen, J., Jørgensen, U., Mortensen, J., Riche, A. B., Schwarz, K.-U., Tayebi, K. and Teixeira, F. (2001). Performance of 15 *Miscanthus* genotypes at five sites in Europe. *Agronomy Journal* **93,** 1013-1019.

Cloutier, Y. and Siminovitch, D. (1982). Correlation between cold-and drought-induced frost hardiness in winter wheat and rye varieties. *Plant Physiology* **69,** 256-258.

Collins, R. P. and Jones, M. B. (1988). The effects of temperature on leaf growth in *Cyperus longus*, a temperate C4 species. *Annals of Botany* **61,** 355-362.

Cooper, J. P. (1964). Climatic variation in forage grasses. I. Leaf development in climatic races of *Lolium* and *Dacylis*. *Journal of Applied Ecology* **1,** 45-61.

Cosgrove, D. (2002). Cell Walls: Structure, Biogenesis, and expansion. In *Plant Physiology.* (ed. L. Taiz and E. Zeiger). Sunderland, Massachusetts: Sinauer Associates.

Cosgrove, D. J. (1981). Analysis of the dynamic and steady state responses of growth rate and turgor pressure to changes in cell parameters. *Plant Physiology* **68.**

Cosgrove, D. J. (1986). Biophysical control of plant cell growth. *Plant Physiology* **37,** 377-405.

Cosgrove, D. J. (1987). Wall relaxation in growing stems: comparison of four species and assessment of measurement techniques. *Planta* **171,** 266-278.

Cosgrove, D. J. (1988). Mechanism of rapid suppression of cell expansion in cucumber hypocotyls after blue-light irradiation. *Planta* **176,** 109-116.

Cosgrove, D. J. (1989). Characterization of Long-term extension of isolated cell walls from growing cucumber hypocotyls. *Planta* **177,** 121-130.

Cosgrove, D. J. (1993a). Wall Extensibility: Its nature measurement and relationship to plant cell wall growth. *New Phytologist* **124,** 1-23.

Cosgrove, D. J. (1993b). Water-uptake by growing plant cells: An assessment of the controlling roles of wall relaxation, solute uptake, and hydraulic conductance. *International Journal of Plant Science* **154,** 10-21.

Cosgrove, D. J. (1997). Assembly and enlargement of the primary cell wall in plants. *Annual Review of Cell and Developmental Biology* .

Cosgrove, D. J. (1999). Enzymes and other agents that enhance cell wall extensibility. *Annual Review of Plant Physiology and Plant Molecular Biology* **50,** 391-417.

Cosgrove, D. J. (2000a). Expansive growth of plant cell walls. *Plant Physiology and Biochemistry* **38.**

Cosgrove, D. J. (2000b). Loosening of plant cell walls by expansins. *Nature* **407.**

Cosgrove, D. J. and Cleland, R. E. (1983a). Osmotic properties of pea internodes in relation to growth and auxin action. *Plant Physiology* **72,** 332-338.

Cosgrove, D. J. and Cleland, R. E. (1983b). Wall relaxation and the driving forces for cell expansive growth. *Plant Physiology* **84.**

Cosgrove, D. J. and Li, Z.-C. (1993). Role of expansin in cell enlargement of oat coleoptiles: Analysis of developmental gradients and photocontrol. *Plant Physiology* **103,** 1321-1328.

Cosgrove, D. J., Patricia, B. and Durachko, D. M. (1997). Group 1 allergens of grass pollen as cell wall loosening agents. *Proceedings of the National Academy of Science USA* **94,** 6559-6564.

Cosgrove, D. J. and Sovonick-Danford, S. (1989). Mechanism of gibberellin-dependent stem elongation in Peas. *Plant Physiology* **89,** 184-191.

Cramer, G. (2003). Differential effects of salinity on leaf elongation kinetics of three grass species. *Plant and Soil* **253,** 233-244.

Cramer, G. R. and Bowman, D. C. (1991). Kinetics of maize leaf elongation. 1. Increased yield threshold limits short-term, steady state elongation rates after exposure to salinity. *Journal of Experimental Botany* **42,** 1417-1426.

Crawford, R. and Huxter, T. (1977). Root growth and carbohydrate metabolism at low temperatures. *Journal of Experimental Botany* **28,** 917-925.

Davies, P. J. (2002). Gibberellins: regulators of plant height. In *Plant Physiology.* (ed. L. Taiz and E. Zeiger). Sunderland, Massachusetts: Sinauer Associates.

Deuter, M. and Abraham, J. (1998). Genetic resources of *Miscanthus* and their use in breeding. In *Biomass for Energy and Industry - Proceedings of the 10th European Bioenergy Conference* (ed. H. Kopetz, T. Weber, W. Palz, P. Chartier and G. L. Ferrero). Würzburg: C.A.R.M.E.N.

Durand, J.-L., Onillon, B., Schnyder, H. and Rademacher, I. (1995). Drought effects on cellular and spatial parameters of leaf growth in tall fescue. *Journal of Experimental Botany* **46,** 1147-1155.

Eagles, C. F. and Othman, O. B. (1981). Growth at low temperature and cold hardiness in white clover. In *Plant Physiology and herbage production , Occasional Symposium No. 13* (ed. C. E. Wright). Hurley, Uk: British Grassland Society.

Earnshaw, M. J., Carver, K. A., Gunn, T. C., Kerenga, K., Harvey, V., Griffiths, H. and Broadmeadow, M. S. J. (1990). Photosynthetic pathway, chilling tolerance and cell sap osmotic potential values of grasses along an altitude gradient in Papua New Guinea. *Oecologia* **84,** 280-288.

Edelmann, H. G., Bergfeld, R. and Schopfer, P. (1989). Role of cell wall biogenesis in the initiation of auxin mediated growth in coleoptiles of *Zea mays* L. *Planta* **179,** 486-94.

Etherington, J. R. (1982). Environment and Plant Ecology. Chichester: John Wiley & Sons.

Fadeel, A., Moll, B. A. and Jones, R. L. (1980). Effect of temperature on the synthesis and secretion of a-amylase in barley aleurone layers. *Plant Physiology* **66,** 466-470.

Farrar, J. F. (1988). Temperature and the partitioning and translocation of carbon. In *Plants and Temperature* (ed. S. P. Long and F. I. Woodward). Cambridge: Company of Biologists.

Fitter, A. H. and Hay, R. K. (2002). Environmental Physiology of Plants. London: Academic Press.

Fowler, D. B. and Limin, A. E. (1997). Breeding for winter hardiness in cereals. In *Proceedings of the International Symposium on Cereal Adaptation to Low Temperature Stress in Controlled Environments* (ed. Z. Bedo, J. Sutka, O. Tischner and B. Köszegi), pp. 169-176. Martonvasar, Hungary: Agricultural research Institute of the Hungarian Academy of Sciences.

Fricke, W. (2002). Biophysical limitation of leaf cell elongation in source-reduced barley. *Planta* **215,** 327-338.

Fry, S. (1995). Polysaccharide-modifying enzymes in plant cell wall. *Annual Review of Plant Physiology and Plant Molecular Biology* **46,** 497-520.

Fry, S. C., Smith, R. C., Renwick, K. F., Martin, D. J., Hodge, S. K. and Matthews, K. J. (1992). Xyloglucan endotransglycosylase, a new wall-loosening enzyme activity from plants. *Biochemical Journal* **282,** 821-828.

Fu, X., Richards, D. E., Ait-ali, T., Hynes, L. W., Ougham, H., Peng, J. and Harberd, N. P. (2002). Gibberellin-Mediated Proteasome-Dependent Degradation of the Barley DELLA Protein SLN1 Repressor. *The Plant Cell* **14,** 3191-3200.

Fuller, M. P. and Eagles, C. F. (1978). A seedling test for cold hardiness in *Lolium perenne* L. *Journal of Agricultural Science, Cambridge* **91,** 217-222.

Gallagher, J. N. and Biscoe, P. V. (1979). Field studies of leaf growth 3. Barley leaf extension in relation temperature, irradiance, and water potential. *Journal of Experimental Botany* **30,** 645-655.

Gastal, F., Belanger, G. and lemaire, G. (1992). A model of the leaf extension rate of tall fescue in response to nitrogen and temperature. *Annals of Botany* **70,** 437-442.

Grace, J. (1988). Temperature as a determinant of plant productivity. In *Plants and Temperature* (ed. S. P. Long and F. I. Woodward). Cambridge: Company of Biologists.

Greef, J. M. and Deuter, M. (1993). Syntaxonomy of *Miscanthus x giganteus* GREEF et DEU. *Angewandte Botanik* **67,** 87-90.

Greef, J. M., Deuter, M., Jung, C. and Schondelmaier, J. (1997). Genetic diversity of European *Miscanthus* species revealed by AFLP fingerprinting. *Genetic Resources and Crop Evolution* **44,** 185-195.

Griffith, M. and Yaish, M. W. F. (2004). Antifreeze proteins in overwintering plants: a tale of two activities. *Trends in Plant Science* **9,** 399-405.

Guy, C. L. (1990). Cold acclimation and freezing stress tolerance: Role of protein metabolism. *Annual Review of Plant Physiology and Plant Molecular Biology* **41,** 187-223.

Haughton, P. M., Sellen, D. B. and Preston, R. D. (1968). Dynamic mechanical properties of the cell wall of *Nitella opaca*. *Journal of Experimental Botany* **19,** 1-12.

Hayashi, I., Hishinuma, Y. and Yamasawa, T. (1981). Structure and functioning of *Miscanthus sinensis* grassland in Sugadaria, Central Japan. *Vegetatio* **48,** 17-25.

Hendry, G. (1987). The ecological significance of fructan in a contemporary *flora*. *New Phytologist* **106,** 201-216.

Hoagland, D. R. and Arnon, D. I. (1951). The water culture method for growing plants without soil. California Agricultural Experiment Station Circular, N. 347.

Hodkinson, T. R., Renvoize, S. A. and Chase, M. W. (1997). Systematics in *Miscanthus*. *Aspects of Applied Biology* **49,** 189-198.

Holt, J. and Orcutt, D. (1996). Temperature thresholds for bud sprouting in perennial weeds and seed germination in cotton. *Weed science* **44**.

Hoson, T., Soga, K. and Mori, R. (2002). Stimulation of elongation growth and cell wall loosening in rice coleoptiles under microgravity conditions in space. *Plant Cell Physiology* **43,** 1067-1071.

Hughes, M. A. and Dunn, M. A. (1996). The molecular biology of plant acclimation to low temperature. *Journal of Experimental Botany* **47,** 291-305.

Humphreys, M. (1989). Assessment of perennial ryegrass (*Lolium perenne* L.) for breeding. II Components of winter hardiness. *Euphytica* **41,** 99-106.

Ivory, D. A. and Whiteman, P. C. (1978). Effects of environment and plant factors on foliar freezing resistance in tropical II Comparison of frost resistance between cultivars of *Cenchrus ciliaris*, *Chloris gayana* and *Setaria anceps*. *Australian Journal of Agricultural Research* **29,** 261-266.

Jones, M. B. and Walsh, M. (2001). *Miscanthus* - for Energy and Fibre. London: James and James (Science Publishers).

Jorgensen, U. and Muhs, H. J. (2001). *Miscanthus* Breeding and Improvement. In *Miscanthus - for Energy and Fibre* (ed. M. B. Jones and M. Walsh). London: James and James (Science Publishers).

Junttila, O., Sevenning, M. M. and Solheim, B. (1990). Effects of temperature and photoperiod on vegetative growth of white clover (*Trifolium repens*) ecotypes. *Physiologia Plantarum* **79,** 427-434.

Kemp, D. R. (1980). The location and size of the extension zone of emerging wheat leaves. *New phytologist* **84,** 229-237.

Kjeldsen, J. B., Jørgensen, U. and Kristensen, E. F. (1999). *Miscanthus* for thatching. In *Markbrug*, vol. 14. Foulum: DJF Rapport.

Kleinendorst, A. (1975). An explosion of leaf growth after stress conditions. *Netherlands Journal of Agricultural Science* **23,** 139-144.

Kleinendorst, A. and Brouwer, R. (1970). The effect of temperature of the root medium and of the growing point of the shoot on growth, water content and sugar content of maize leaves. *Netherlands Journal of Agricultural Science* **18,** 140-148.

Kleinendorst, A. and Brouwer, R. (1972). The effects of local cooling on growth and water content of plants. *Netherlands Journal of Agricultural Science* **20,** 203-217.

Korner, C. and Larcher, W. (1988). Plant life in cold climates. In *Plants and Temperature* (ed. S. P. Long and F. I. Woodward). Cambridge: Company of Biologists.

Korner, C. and Woodward, F. (1987). The dynamics of leaf extension in plants with diverse altitudinal ranges 2. field studies between 600 and 3200 m altitude. *Oecologia* **72,** 279-283.

Kutschera, U. and Briggs, W. (1988). Growth, *in vivo* extensibility, and tissue tension in developing pea internodes. *Plant Physiology* **86,** 306-311.

Kutschera, U. and Briggs, W. R. (1987). Differential effect of auxin on *in vivo* extensibility of cortical cylinder and epidermis in pea internodes. *Plant Physiology* **84,** 1361-1366.

Kutschera, U. and Schopfer, P. (1986a). Effect of auxin and abscisic acid on cell wall extensibility in maize coleoptiles. *Planta* **167,** 527-535.

Kutschera, U. and Schopfer, P. (1986b). In-vivo measurement of cell-wall extensibility in maize coleoptile: effects of auxin and abscisic acid. *Planta* **169,** 437-442.

Lanahan, M. B. and Tuan-Hua, D. H. (1988). Slender barley: A constitutive gibberellin-response mutant. *Planta* **175,** 107-114.

Larcher, W. (2002). Physiological Plant Ecology. Berlin: Springer-Verlag.

Levitt, J. (1980). Response of Plants to Environmental Stress Volume I & II. London: Academic Press.

Lewandowski, I. (1998). EMI: European Miscanthus Improvement - The results from the first year of a Miscanthus Breeding Project. In *Biomass for Energy and Industry - Proceedings of the 10th European Bioenergy Conference* (ed. H. Kopetz, T. Weber, W. Palz, P. Chartier and G. L. Ferrero). Würzburg: C.A.R.M.E.N.

Lewandowski, I., Clifton-Brown, J. C., Andersson, B., Basch, G., Tayebi, K., Christian, D. G., Deuter, M., Jørgensen, U., Bonderup-Kjeldsen, J. and Schwarz, K.-U. (2000a). Biomass potential of *Miscanthus* genotypes in Europe - Results of European wide screening. In *3rd International Crop Science Congress*, pp. 17 - 22 August 2000, p 199. Hamburg, Germany.

Lewandowski, I., Clifton-Brown, J. C., Scurlock, J. M. O. and Huisman, W. (2000b). *Miscanthus*: European experience with a novel energy crop. *Biomass and Bioenergy* **19,** 209-277.

Lewandowski, I. and Kicherer, A. (1997). Combustion quality of biomass: practical relevance and experiments to modify the biomass quality of *Miscanthus x giganteus*. *European Journal of Agronomy* **6,** 163-177.

Lockhart, J. A. (1965). An analysis of irreversible plant cell elongation. *Journal of Theoretical Biology* **8,** 264-275.

Long, S. P. and Beale, C. V. (2001). Resource utilisation by *Miscanthus*. In *Miscanthus - for Energy and Fibre* (ed. M. B. Jones and M. Walsh). London: James and James (Science Publishers).

Masuda, Y. (1990). Auxin-induced cell wall loosening. *Botanical Magazine of tokyo* **103,** 345-370.

Mattews, M. A., Volkenburgh, E. V. and Boyer, J. (1984). Acclimation of leaf growth to low water potential in sunflower. *Plant Cell and Environment* **7,** 199-206.

Matumura, M., Hasegawa, T. and Saijoh, Y. (1985). Ecological aspects of *Miscanthus sinensis* var. condensatus, *M. x sacchariflorus*, and their 3x-, 4x-hybrids. 1. Process of vegetative spread. *Research Bulletin of the Faculty of Agriculture, Gifu University* **50,** 423-433.

McCann, M. (2001). Approaches to understanding the functional architecture of the plant cell wall. *Phytochemistry* **57,** 811-821.

McNeil, S., Nuccio, M. and Hanson, A. (1999). Betamines and related osmoprotectants: targets for metabolic engineering of stress resistance. *Plant Physiology* **120,** 945-949.

McQueen-Mason, S. and Cosgrove, D. J. (1992). Two Endogenous Proteins that induce cell Wall Extension in plants. *The Plant Cell* **4,** 1425-1433.

McQueen-Mason, S. J. (1995). Expansins and cell wall expansion. *Journal for Experimental Botany* **46,** 1639-1650.

Miedema, P. (1982). The effects of low temperature on *Zea mays*. *Advances in Agronomy* **35,** 93-128.

Miedema, P., post, J. and Groot, P. (1987). The effects of low temperature on seedling growth of maize genotypes. In *PUDOC*: Wageningen.

Monteith, J. L. (1977). Climate and the efficiency of crop production in Britain. *Philosophical Transactions of the Royal Society* **B281,** 277-294.

Nakamura, Y., Wakabayashi, K. and Hoson, T. (2003). Temperature modulates the cell wall mechanical properties of rice coleoptiles by altering the molecular mass of hemicellulosic polysaccharides. *Physiologia Plantarum* **118,** 597-604.

Nakamura, Y., Wakabayashi, K., Kamisaka, S. and Hoson, T. (2002). Effects of temperature on the cell wall and osmotic properties in dark-grown and azuki bean seedlings. *Journal of Plant Research* **115,** 455-461.

Neighbour, E. A., Pearson, M., Paul, N. D., Wood, W. A., Smith, P. J., Johnston, G. K. and Caporn, S. J. M. (1990). A small-scale controlled environment chamber for the investigation of effects of pollutant gases on plants growing at cool or sub-zero temperature. *Environmental Pollution* **64,** 155-168.

Nobel, P. S. (1988). Principles underlying the prediction of temperature in plants, with special reference to desert succulents. In *Plants and Temperature* (ed. S. P. Long and F. I. Woodward). Cambridge: Company of Biologists.

Nonami, H. and Boyer, J. S. (1990). Wall Extension and cell hydraulic conductivity decrease in enlarging stem tissues at low water potentials. *Plant Physiology* **193,** 1610-1619.

Numata, M. (1974). Grassland Vegetation. In *The Flora and Vegetation of Japan* (ed. M. Numata). Tokyo: Elsevier.

Numata, M. and Mitsudera, M. (1969). Efficient environmental factors to the growth and production of *Miscanthus sinensis* grasslands in Japan. *Japanese Journal of Botany* **20,** 135-151.

Ong, C. K. and Baker, C. K. (1985). Temperature and leaf growth. In *Control of Leaf Growth* (ed. N. R. Baker, W. J. Davies and C. K. Ong). Cambridge: Cambridge University Press.

Ostgard, O. and Eagles, C. F. (1971b). Variation in growth and development in natural populations of *Dactylis glomerata* from Norway and Portugal. 1I. Leaf development and tillering. *Journal of Applied Ecology* **8,** 383-391.

Palta, J. P., Whitaker, B. D. and Weiss, L. S. (1993). Plasma membrane lipids associated with genetic variability in freezing tolerance and cold acclimation of *Solanum* species. *Plant Physiology* **103,** 793-803.

Pardossi, A., Pritchard, J. and Tomos, A. D. (1994). Leaf illumination and root cooling inhibit bean leaf expansion by decreasing turgor pressure. *Journal of Experimental Botany* **45,** 415-422.

Parsons, A. J. and Robson, M. J. (1980). Seasonal changes in the physiology of S.24 perennial ryegrass (*Lolium perenne* L.). I. Response of leaf extension to temperature during the transition from vegetative to reproductive growth. *Annals of Botany* **46,** 435-445.

Parvez, M. M., Wakabayashi, K., Hoson, T. and Kamisaka, S. (1996). Changes in cellular osmotic potential and mechanical properties of cell walls during light-induced inhibition of cell elongation in maize coleoptiles. *Physiologia Plantarum* **96,** 179-185.

Passioura, J. B. (1994). The physical chemistry of the plant cell wall: implications for the control of expansion rate. *Journal of Experimental Botany* **45,** 1675-1682.

Peacock, J. M. (1975a). Temperature and leaf growth in *Lolium perenne* 1. The thermal microclimate: its measurement and relation to crop growth. *Journal of Applied Ecology* **12,** 99-114.

Peacock, J. M. (1975b). Temperature and leaf growth in *Lolium perenne* 2. The site of temperature perception. *Journal of Applied Ecology* **12,** 115-123.

Peacock, J. M. (1976). Temperature and leaf growth in four grass species. *Journal of Applied Ecology* **13,** 225-232.

Pollock, C. J. (1982). Responses and tolerance of *Sorghum* to temperature stress. In *Sorghum in the Eighties* (ed. L. House, L. K. Mughogho and J. M. Peacock). Pantacheru, India: ICRISAT.

Pollock, C. J. and Eagles, C. F. (1988). Low temperature and the growth of plants. In *Plants and Temperature* (ed. S. P. Long and F. I. Woodward). Cambridge: Company of Biologists.

Pollock, C. J., Eagles, C. F., Howarth, C. J., Schunmann, P. H. D. and Stoddart, J. L. (1993a). Temperature stress. In *Plant adaptation to environmental stress* (ed. L. Fourden, T. A. Mansfield and J. Stoddart). London: Chapman Hall.

Pollock, C. J., Lloyd, E. J., Stoddart, J. L. and Thomas, H. (1983). Growth, photosynthesis and assimilate partitioning in *Lolium temulentum* exposed to chilling temperatures. *Physiologia Plantarum* **59,** 257-262.

Pollock, C. J., Thomas, H. and Stoddart, J. L. (1993b). Temperature and the control of leaf extension in temperate grasses and cereals. In *Proceedings of COST 814: crop adaptation to cool wet climates.* (ed. T. H. Wilson D, Pithan K). Brussels: Commission of the European Communities.

Pollock, C. J., Tomos, A. D., Thomas, A., Smith, C. J., Lloyd, E. J. and Stoddart, J. L. (1990). Extension growth in a barley mutant with reduced sensitivity to low temperature. *New Phytologist* **115,** 617-623.

Potter, J. R. and Jones, J. W. (1977). Leaf area partitioning as an important factor in growth. *Plant Physiology* **59,** 10-14.

Pritchard, J. (1994). The control of cell expansion in roots. *New Phytology* **127,** 3-26.

Pritchard, J., Barlow, P. W., Adams, J. S. and Tomos, A. D. (1990a). Biophysics of the inhibition of the growth of maize roots by lowered temperature. *Plant Physiology* **93,** 222-230.

Pritchard, J., Jones, R. G. W. and Tomos, A. D. (1990b). Measurement of yield threshold and cell wall extensibility of intact wheat roots under different ionic, osmotic and temperature treatments. *Journal of Experimental Botany* **41,** 669-675.

Proseus, T. E., Zhu, G.-L. and Boyer, J. S. (2000). Turgor, temperature and the growth of plant cells: using Chara *corallina* as a model system. *Journal of Experimental Botany* **51,** 1481-1494.

Rajashekar, C. B. and Lafta, A. (1996). Cell-wall changes and cell tension in response to cold acclimation and exogenous abscisic acid in leaves and cell cultures. *Plant Physiology* **111,** 605-612.

Rapacz, M. (1998). Physiological effects of winter rape (*Brassica napus* var. *oleifera*) prehardening to frost. II. Growth, energy partitioning and water status during cold acclimation. *Journal of Agronomy and Crop Science* **181,** 81-87.

Ray, P. M. and Ruesink, M. (1962). Kinetic experiments on the nature of the growth mechanism in oat coleoptile cells. *Development Biology* **4,** 377-97.

Rees, T. A., Dixon, W. L., Pollock, C. J. and Franks, F. (1982). Low temperature sweetening of higher plants. In *Recent Advances in the Biochemistry of Fruit and Vegetables* (ed. J. Friend and M. J. C. Rhodes). London: Academic Press.

Rees, T. A. P., Burrell, M. M., Entwhistle, T. G., Hammond, J. B. W., Kirk, D. and Kruger, N. J. (1988). Effects of low temperature on the respiratory metabolism of carbohydrates by plants. In *Plants and Temperature* (ed. S. P. Long and F. I. Woodward). Cambridge: Company of Biologists.

Revilla, P., Malvar, R. A., Cartea, M. E., Butron, A. and Ordas, A. (2000). Inheritance of cold tolerance at emergence and during early season growth in maize. *Crop Science* **40,** 1579-1585.

Richards, D. E., King, K. E., Ait-ali, T. and Harberd, N. P. (2001). How gibberellin regulates plant growth and development: A molecular genetic analysis of gibberellin signalling. *Annual Review of Plant Physiology and Plant Molecular Biology* **52,** 67-88.

Roberts, E. H. (1988). Temperature and seed germination. In *Plants and Temperature* (ed. S. P. Long and F. I. Woodward). Cambridge: Company of Biologists.

Robson, M. J. and Jewiss, O. R. (1968a). A comparison of British and north African varieties of tall fescue (*Festuca arundinacea*) II growth during winter and survival at low temperature. *Journal of Applied Ecology* **5,** 182-191.

Robson, M. J. and Jewiss, O. R. (1968b). A comparison of British and north African varieties of tall fescue (*Festuca arundinacea*) III effects of light, temperature and day length on relative growth rate and its components. *Journal of Applied Ecology* **5,** 191-204.

Satorre, E., Rizo, F. and Arias, S. (1996). The effect of temperature on sprouting and early establishment of *Cyndon dactylon*. *Weed Research* **36,** 431-440.

Scally, L., Hodkinson, T. and Jones, M. B. (2001). Origins and taxonomy of *Miscanthus*. In *Miscanthus - for Energy and Fibre* (ed. M. B. Jones and M. Walsh). London: James and James (Science Publishers).

Schnyder, H. and Nelson, C. J. (1988). Diurnal growth of tall fescue blades 1. Spatial distribution of growth, deposition of water, and assimilate import in the elongation zone. *Plant Physiology* **86,** 1070-1076.

Schunmann, P., Ougham, H. and Turk, K. (1994). Leaf extension in the slender barley mutant: delineation of the zone of cell expansion and changes in translatable mRNA during leaf development. *Plant Cell and Environment* **17,** 1315-1322.

Schunmann, P. H. D., Smith, R. C., Lang, V., Matthews, P. R. and Chandler, P. M. (1997). Expression of XET-related genes and its relation to elongation in leaves of barley (*Hordeum vulgare* L.). *Plant, Cell and Environment* **20,** 1439-1450.

Seefeldt, S. S., Kidwell, K. K. and Waller, J. E. (2002). Base growth temperatures, germination rates and growth response of contemporary spring wheat (*Triticum aestivum* L.) cultivars from the US Pacific Northwest. *Field Crop Research* **75,** 47-52.

Sellen, D. B. (1980). The mechanical properties of plant cell walls. In *The mechanical properties of biological materials* (ed. J. F. V. Vincent and J. D. Curry): Cambridge.

Shackel, K. A., Matthews, M. M. and Morrison, J. C. (1987). Dynamic relation between expansion and cellular turgor in growing grape (*Vitis vinifera L.*) leaves. *Plant Physiology* **84,** 1166-1171.

Showalter, A. (1993). Structure and Function of Cell Wall Proteins. *The plant cell* **5,** 9-23.

Smith, R. C., Matthews, P. R., Schunmann, P. H. D. and Chandler, P. M. (1996). The regulation of leaf elongation end xyloglucan endotransglycosylase by gibberellin in 'Himalaya' barley (*Hordeum vulgare* L.). *Journal of Experimental Botany* **47,** 1395-1404.

Soga, K., Harada, K., Wakabayashi, K., Hoson, T. and Kamisaka, S. (1999). Increased molecular mass of hemicellulosic polysaccharides is involved in growth inhibition of maize coleoptiles and mesocotyls under hypergravity conditions. *Journal of Plant Research* **112,** 273-278.

Somerville, C., Browse, J., Jawororski, J. G. and Ohlrogge, J. B. (2000). Lipids. In *Biochemistry and Molecular Biology of Plants* (ed. B. B. Buchanan, W. Gruissem and R. J. Jones). Rickville, Maryland: American Society of Plant Physiologists.

Spollen, W. G., R.E.Sharp, Saab, I. N. and Wu, Y. (1993). Regulation of cell expansion in roots and shoots at low water potentials. In *Water deficits: plant responses from cell to community* (ed. J. A. C. Smith and H. Griffiths). Oxford: Bios.

Steinmaus, S. J., Prather, T. S. and Holt, J. S. (2000). Estimation of base temperatures for nine weed species. *Journal of Experimental Botany* **51,** 275-286.

Steudle, E. (1985). Water transport as a limiting factor in extension growth. In *Control of leaf growth* (ed. N. R. Baker, W. J. Davies and C. K. Ong): Cambridge University Press.

Stoddart, J. L. and Lloyd, E. J. (1986). Modification by gibberellin of the growth-temperature relationship in mutant and normal genotypes of several cereals. *Planta* **167,** 364-368.

Stoddart, J. L., Thomas, H., Lloyd, E. J. and Pollock, C. J. (1986). The use of temperature-profiled position transducer for the study of low-temperature growth in Gramineae. Equipment design and output interpretation. *Planta* **167,** 359-363.

Stushnoff, C., Fowler, B. and Bruele-Babel, A. (1984). Breeding and selection for resistance to low temperature. In *Crop Breeding - a contemporary basis* (ed. P. B. Vose and S. G. Blixt). Oxford: Pergamon Press.

Sutcliffe, J. (1977). Plants and Temperature. London: Edward Arnold.

Svenning, M. M., Røsnes, K. and Junttila, O. (1997). Frost tolerance and biochemical changes during hardening and dehardening in contrasting white clover populations. *Physiologia Plantarum* **101,** 31-37.

Svenning, M. M., Sandli, N. and Juttila, O. (1993). Development of frost hardiness in white clover (*Trifolium repens*) as affected by temperature and nitrogen. In *Proceedings of COST 814: crop adaptation to cool wet climates.* (ed. T. H. Wilson D, Pithan K). Brussels: Commission of the European Communities.

Taiz, l. (1984). Plant cell expansion: Regulation of cell wall mechanical properties. *Annual Review of Plant Physiology* **35,** 585-657.

Taiz, L. and Zeiger, E. (2002). Plant Physiology. Sunderland, Massachusetts: Sinauer Associates.

Taylor, G. and Davies, W. J. (1986). Yield turgor of growing leaves of *Betula* and *Acer*. *New Phytologists* **104,** 347-353.

Thomas, A. (1990). Low temperature growth in forage grasses and cereals. Cardiff.

Thomas, A., Tomos, A. D., Stoddart, J. L., Thomas, H. and Pollock, C. J. (1989). Cell expansion rate, temperature and turgor pressure in growing leaves of *Lolium temulentum* L. *New Phytologist* **112,** 1-5.

Thomas, H. (1983). Analysis of the response of leaf extension to chilling temperatures in *Lolium temulentum* seedlings. *Physiologia Plant* **57,** 509-513.

Thomas, H., James, A. and Kamau, J. (1993). Co-acclimation to cold, heat and drought in perennial ryegrass and maize. In *Proceedings of COST 814: crop adaptation to*

cool wet climates. (ed. T. H. Wilson D, Pithan K). Brussels: Commission of the European Communities.

Thomas, H., James, A. R. and Humphreys, M. (1999). Effects of water stress on leaf growth in tall fescue, Italian ryegrass and their hybrid: rheological properties of expansion zones of leaves, measured on growing and killed tissue. *Journal of Experimental Botany* **50,** 221-231.

Thomas, H. and Norris, I. B. (1977). The growth responses of *Lolium perenne* to the weather during winter and spring at various altitudes in mid-Wales. *Journal of Applied Ecology* **14,** 949-964.

Thomas, H. and Potter, J. F. (1985). Fitting logistic -type curves to extension growth data for leaves of grass species by means of the Maximum Likelihood Program: analysis of leaf extension in *Lolium temulentum* at optimal and chilling temperatures. *Environmental and Experimental Botany* **25,** 157-163.

Thomas, H. and Stoddart, J. L. (1984). Kinetics of leaf growth in *Lolium temulentum* at optimal and chilling temperatures. *Annals of Botany* **53,** 341-347.

Thomas, H. and Stoddart, J. L. (1995). Temperature sensitivities of *Festuca arundinacea* Schreb. and *Dactylis glomerata* L. ecotypes. *New Phytologist* **130,** 125-134.

Thomashaw, M. (2001). So what's new in the field of plant cold acclimation? Lots! *Plant Physiology* **125,** 89-93.

Thomashow, M. (1999). Plant Cold Acclimation: Freezing Tolerance Genes and Regulatory Mechanisms. *Annual Review of Plant Physiology and Plant Molecular Biology* **50,** 571-599.

Tiryaki, I. and Andrews, D. (2001a). Germination and seedling cold tolerance in Sorghum: I. Evaluation of rapid screening methods. *Agronomy Journal* **93,** 1386-1391.

Tomos, A. D. (1985). The Physical limitations of leaf cell expansion. In *Control of leaf growth. SEB Seminar Series 27* (ed. N. R. Baker, W. J. Davies and C. K. Ong): Cambridge University press.

Tomos, D. (1999). The Pressure Probe: A versatile tool in plant cell physiology. *Annual Review of Plant Physiology and Plant Molecular Biology* **50,** 447-472.

Tonkinson, C. L., Lyndon, R. F., Arnold, G. M. and Lenton, J. R. (1997). The effects of temperature and the *Rht3* dwarfing gene on growth, cell extension, and gibberellin content and responsiveness in the wheat leaf. *Journal of Experimental Botany* **48,** 963-970.

Visser, P. and Pignatelli, V. (2001). Utilisation of *Miscanthus*. In *Miscanthus - for Energy and Fibre* (ed. M. B. Jones and M. Walsh). London: James and James (Science Publishers).

Wagenaar, B. M. and Vandenheuvel, E. J. M. T. (1997). Co-combustion of Miscanthus in a pulverised coal combustor - experiments in a drop tube furnace. *Biomass and Bioenergy* **12,** 185-197.

Watts, W. R. (1972a). Leaf extension in *Zea mays* 1. Leaf extension and water potential in relation to root zone and air temperatures. *Journal of Experimental Botany* **23,** 704-712.

Watts, W. R. (1972b). Leaf extension in *Zea mays* 2. Leaf extension in response to independent variation of the temperature of the apical meristem, of the air around the leaves, and of the root zone. *Journal of Experimental Botany* **23,** 713-721.

Weiser, R. L., Wallner, S. J. and Waddell, J. W. (1990). Cell wall and extensin mRNA changes during cold acclimation of pea seedlings. *Plant Physiology* **93,** 1021-1026.

Wen, C. K. and Chang, C. (2002). Arabidopsis RGL1 encodes a negative regulator of gibberellin responses. *Plant Cell* **14,** 87-100.

Weng, J.-H. and Ueng, R.-G. (1997). Effect of temperature on photosynthesis of Miscanthus clones collected from different elevations. *Photosynthetica* **34,** 307-311.

Whitney, S. E. C., Gidley, M. J. and McQueen-Mason, S. J. (2000). Probing expansin action using cellulose/hemicellulose composites. *The Plant Journal* **22,** 327-334.

Wiese, A. and Binning, L. (1987). Calculating the threshold temperature of development for weeds. *Weed Science* **35,** 177-179.

Williams, J. P., Khan, M. U., Mitchell, K. and Johnson, G. (1988). The effect of temperature on the level and biosynthesis of unsaturated fatty acids in diacylglycerols of *Brassica napus* leaves. *Plant Physiology* **87,** 904-910.

Woodward, F. I. (1988). Temperature and the distribution of plant species and vegetation. In *Plants and Temperature* (ed. S. P. Long and F. I. Woodward). Cambridge: Company of Biologists.

Woodward, F. I. and Friend, A. D. (1988). Controlled environment studies on the temperature responses of leaf extension in species of *Poa* with diverse altitudinal ranges. *Journal of Experimental Botany* **39,** 411-430.

Woodward, F. I., Körner, C. and Crabtree, R. C. (1986). The dynamics of leaf extension in plants with diverse altitudinal ranges. *Oecologia* **70,** 222-226.

Wu, J., Lightner, J., Warwick, N. and Browse, J. (1997). Low-temperature damage and subsequent recovery of *fab1* mutant Arabidopsis exposed to 2 ^{o}C. *Plant Physiology* **113,** 347-356.

Wu, Y. and Cosgrove, D. (2000). Adaptation of roots to low water potentials by changes in cell wall extensibility and cell wall proteins. *Journal of Experimental Botany* **51,** 1543-1553.

Wu, Y., Sharp, R. E., Durachko, D. M. and Cosgrove, D. J. (1996). Growth maintenance of the maize primary root at low water potentials involves increases in cell-wall extension properties, expansin activity, and wall susceptibility to expansins. *Plant Physiology* **111,** 765-772.

Wu, Y., Spollen, W. G., Sharp, R. E., Hetherington, P. R. and Fry, S. C. (1994). Root growth maintenance at low water potentials. Increased activity xyloglucan endotransglycosylase and its possible regulation by abscisic acid. *Plant Physiology* **106,** 607-615.

Zabotin, A. I., Barisheva, T. S., Zabotina, O. A., Larskaya, I. A., Lozovaya, V. V., Beldman, G. and Voragen, A. G. L. (1998). Alterations in cell walls of winter wheat roots during low temperature acclimation. *Journal of Plant Physiology* **152,** 473-479.

Ziegenhagen, B., Junge, R. and Muhs, H.-J. (1995). Effects of frost temperatures on early growth of *Miscanthus* "Giganteus". In *Biomass for energy, environment, agriculture and industry*, vol. 1 (ed. P. Chartier, A. A. C. M. Beenackers and G. Grassi), pp. 565-574. Oxford: Elsevier.

Zurek, D. M., Rayle, D. L., Morris, T. C. M. and Clouse, S. D. (1994). Investigation of gene expression, growth kinetics, and wall extensibility during Brassinosteroid-regulated stem elongation. *Plant Physiology* **104,** 505-513.